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Displacement fractionation of deoxyribonucleoproteins by heparin and dextran sulphate. By P. W. Kent, M. Hichens and P. F. V. Ward

Calcium, magnesium and phosphorus in foetal tissues. By Cleopatra Economou-Mavrou and R. A. McCance

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75. Further observations on the metabolism of hydrazides of aromatic acids. By A. M. El Masri, J. N. Smith and R. T. Williams

The vitamin B_{12}-binding systems of isolated intestine of the rat. By A. L. Latner and Laureen C. D. P. Raine

The proteolytic activity of the venom of *Bothrops jararaca*. By Olga B. Henriques, Alba A. C. Lavras, Mina Fichman, Fajga R. Mandelbaum and S. B. Henriques

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Determination of amino acids by double isotope-dilution technique. By J. K. Whitehead

The reaction of pentoses with anthrone. By R. W. Bailey

Effect of amino acids on an anaerobic phosphate-exchange reaction in *Escherichia coli*. By L. V. Eggleston

Effect of ischaemic necrosis and autolysis *in vitro* on the soluble proteins of rat kidneys. By R. Caravaglios

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The oligosaccharides of human milk. By F. H. Malpress and F. E. Hytten

Preparation of crystalline porphyrin esters from bovine porphyria urine. By T. K. With

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CORRIGENDA

Liver and brain mitochondria

By W. N. Aldridge

Volume 67 (1957)

p. 425, col. 1, line 20 and col. 2, line 12:

for 1500 rev./min. *read* 1100 rev./min.

Toxicity of autoxidized squalene and linoleic acid, and of simpler peroxides, in relation to toxicity of radiation

By V. J. Horgan, J. St L. Philpot, Barbara W. Porter & D. B. Roodyn

Volumn 67 (1957)

p. 554, Fig. 1, ordinate scale:

This should read $-0{\cdot}5$, $-1{\cdot}5$ *and* $-2{\cdot}5$ *downwards; delete* 1×10^{-3}

A comparison of the properties of mitochondria isolated from liver and heart

By F. A. Holton, W. C. Hülsmann, D. K. Myers & E. C. Slater

Volume 67 (1957)

p. 584, col. 1, legend to Fig. 4:

line 7: *for* ●—● *read* ●- - -●

line 13: *for* ●- - -● *read* ●—●

The purification and properties of rabbit-muscle myokinase

By O. H. Callaghan

Volume 67 (1957)

p. 653, col. 2, line 12 from end:

for $E_{280\,m\mu}/E_{260\,m\mu} = 1{\cdot}28$ *read* $E_{280\,m\mu}/E_{260\,m\mu} = 1{\cdot}98$

Studies on the biosynthesis of riboflavin

4. Purine metabolism and riboflavin synthesis in *Eremothecium ashbyii*

By E. G. Brown, T. W. Goodwin & O. T. G. Jones

Volume 68 (1958)

p. 40, formula for riboflavin:

for $CH_2 \cdot [CH(OH)] \cdot CH_2 \cdot OH$ *read* $CH_2 \cdot [CH(OH)]_3 \cdot CH_2 \cdot OH$

Isolation of *iso*Xanthopterin from Human Urine

By J. A. BLAIR
University College of Ghana, Ghana, West Africa

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The pterins xanthopterin (2-amino-4:6-dihydroxypteridine) (Koschara, 1936) and biopterin (2-amino-4-hydroxy-6-[1:2-dihydroxypropyl-(L-*erythro*)]-pteridine) (Patterson, Saltza & Stokstad, 1956) have been isolated from human urine. It has been suggested that the pterins 2-amino-4-hydroxypteridine-6-carboxylic acid, 2-amino-4-hydroxypteridine and *iso*xanthopterin (2-amino-4:7-dihydroxypteridine) are products of catabolism of pteroyl-L-glutamic acid (Blair & Graham, 1955). It has also been shown that pteroyl-L-glutamic acid is converted by methylene blue and xanthine oxidase into 2-amino-4-hydroxypteridine-6-carboxylic acid (Blair, 1957). It seemed desirable, therefore, to examine human urine further.

When freshly voided adult male urine was analysed, as described below, a small quantity of a purple fluorescent substance could be isolated. On further purification this substance was shown to be *iso*xanthopterin, by its similar behaviour to an authentic sample in six solvent systems.

No 2-amino-4-hydroxypteridine-6-carboxylic acid could be isolated from freshly voided human urine. If, however, samples were allowed to stand, or if the analysis was unduly protracted, a bright-blue fluorescent substance could be isolated. This was not separable from 2-amino-4-hydroxypteridine-6-carboxylic acid in five different solvent systems. It was assumed that this acid was derived from the decomposition of biopterin in the urine.

The method used was not suitable for the isolation of 2-amino-4-hydroxypteridine.

EXPERIMENTAL

Conc. HCl (25 ml.) was added to adult male urine (250 ml.) which had been freshly voided into a flask containing a trace of phenol. The urine was then extracted with liquid phenol (50 ml.; Cranmer, 1948) and the phenol layer shaken up with water (25 ml.) and ether (100 ml.). The aqueous layer was washed twice with ether (100 ml. portions) and then evaporated to dryness under reduced pressure.

The residue was dissolved in water (5 ml.) and a few drops of aq. NH_3 were added until the solution was alkaline. The solution was filtered and evaporated to dryness under reduced pressure and the residue dissolved in aq. 0·5N-NH_3 (0·5 ml.).

The dark-brown, syrupy liquid was spotted on Whatman no. 3MM paper so that it filled a rectangle 3 in. × 1 in. The paper was then developed (descending, overnight, in darkness) with propanol–5% acetic acid (2:1, v/v), air-dried and viewed in 365 mμ light.

Three fluorescent areas could be seen: nearest the origin, a well-defined purple–blue band (1); next, a bright-yellow band (2); finally, a smaller yellow band (3). After the third yellow band the chromatogram was badly streaked. The first band was cut out and eluted with aq. 0·5N-NH_3. Further purification by chromatography, with no. 3MM paper and *tert.*-butyl alcohol–pyridine–water (50:15:35, by vol.), resolved this into three subsidiary bands. The eluate of the middle band (R_F 0·3–0·4) was chromatographed on no. 1 Whatman paper with propanol–aq. 1% NH_3 (2:1, v/v). Finally the eluate from this last chromatogram was evaporated to dryness under reduced pressure and the residue dissolved in aq. 0·5N-NH_3 (0·05 ml.). This solution was used for identification.

The purple fluorescent substance showed identical chromatographic behaviour on Whatman no. 1 paper with that of an authentic sample of *iso*xanthopterin, in butanol–acetic acid–water (4:1:5, by vol.; Good & Johnson, 1949), propanol–5% acetic acid (2:1, v/v; Forrest & Mitchell, 1954*a*), propanol–1% aq. NH_3 (2:1, v/v; Hadorn & Mitchell, 1951), propanol–N-HCl (2:1, v/v; Forrest & Mitchell, 1954*b*), and *tert.*-butyl alcohol–pyridine–water (50:15:35, by vol.; Forrest & Todd, 1950).

With *tert.*-butyl alcohol–pyridine–water (60:15:25, by vol.; Forrest & Todd, 1950) a difference of 0·06 was found in the R_F values of the unknown and *iso*xanthopterin. A mixture of the unknown and *iso*xanthopterin, however, did not separate into two spots and had a similar R_F to the unknown. This difference in R_F values of the unknown and *iso*xanthopterin is therefore due to the presence of impurities.

An additional amount of *iso*xanthopterin could be obtained by eluting band (2) and purifying it as before. In one experiment band (1) did not appear and *iso*xanthopterin was isolated from band (2).

For each identification 5μl. of solution was used and the dried papers were viewed in 365 mμ light. Papers were also viewed in 254 mμ light. This was less satisfactory as these pterins do not fluoresce so brilliantly as in 365 mμ light.

If the samples of urine were allowed to stand, or if the preliminary stages of the analysis were protracted, an additional bright blue band was found between band (1) and the origin. This band was eluted with aq. 0·5N-NH_3. It was not separable from authentic 2-amino-4-hydroxypteridine-6-carboxylic acid in five solvent systems. It was probably, therefore, 2-amino-4-hydroxypteridine-6-carboxylic acid formed from the decomposition of biopterin.

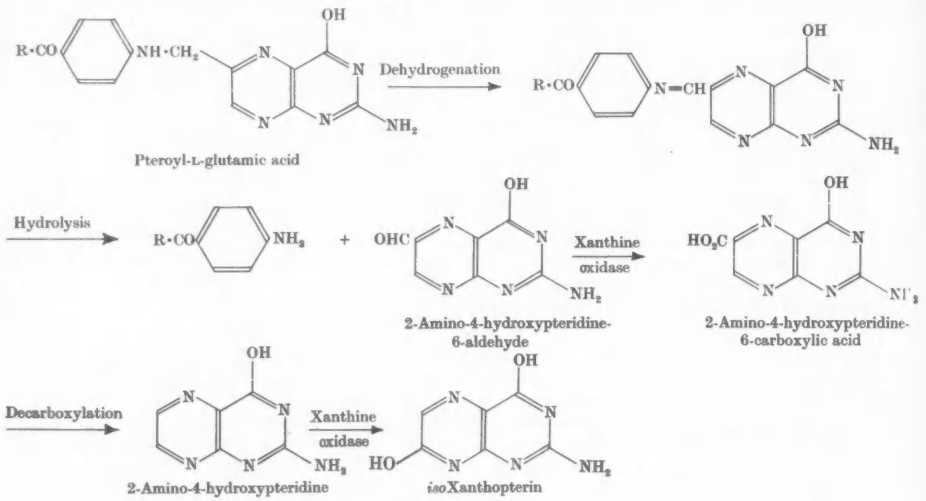

Fig. 1. Catabolism of pteroyl-L-glutamic acid. R represents $HO_2C{\cdot}[CH_2]_2{\cdot}CH(CO_2H){\cdot}NH{\cdot}$.

DISCUSSION

It has been suggested that pteroyl-L-glutamic acid is catabolized in the body by some yet unknown route (Williams, Eakin, Beerstecher & Shive, 1950). It is now suggested that the catabolism of pteroyl-L-glutamic acid proceeds as in Fig. 1.

Evidence for the conversion of pteroyl-L-glutamic acid into 2-amino-4-hydroxypteridine-6-carboxylic acid by processes of dehydrogenation, hydrolysis and enzymic oxidation has been described in a previous paper (Blair, 1957).

The pterins 2-amino-4-hydroxypteridine-6-carboxylic acid, 2-amino-4-hydroxypteridine and *iso*xanthopterin are widely distributed in Nature (Forrest, Van Baalen & Myers, 1957; Viscontini, Kuhn & Egelhaaf, 1956; Forrest & Mitchell, 1955; Viscontini, Schmid & Hadorn, 1955; Viscontini, Loeser, Karrer & Hadorn, 1955; Nawa & Taira, 1954; Hama, 1953; Busnel & Drilhon, 1949; for earlier references see Blair & Graham, 1955).

2-Amino-4-hydroxypteridine-6-carboxylic acid can be decarboxylated by heat (Forrest & Mitchell, 1954*b*) or by ultraviolet light (Lowry, Bessey & Crawford, 1949*a*; Viscontini, Loeser & Egelhaaf, 1956) to 2-amino-4-hydroxypteridine; and this can be oxidized by xanthine oxidase to *iso*xanthopterin (Lowry, Bessey & Crawford, 1949*b*).

It is therefore reasonable to suppose that these three pterins are intermediate in the biological degradation of pteroyl-L-glutamic acid. The isolation of *iso*xanthopterin from human urine supports this scheme. Further work is in progress.

SUMMARY

1. *iso*Xanthopterin has been isolated from human urine.

2. 2-Amino-4-hydroxypteridine-6-carboxylic acid could not be isolated from freshly voided human urine.

3. A scheme for the catabolism of pteroyl-L-glutamic acid is suggested.

We wish to thank the Chemical Society for a grant and Professor D. H. R. Barton, F.R.S., for accommodation during the long vacations in 1956 and 1957.

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Studies on Sulphatases

19. THE PURIFICATION AND PROPERTIES OF ARYLSULPHATASE B OF HUMAN LIVER*

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Evidence is accumulating which suggests that two different types of arylsulphatases occur in Nature. Dodgson (1956) and Dodgson & Spencer (1957*a*) have tentatively suggested that those arylsulphatases which show considerable affinity and activity towards simple arylsulphates such as potassium *p*-nitrophenyl sulphate (NPS) and potassium *p*-acetylphenyl sulphate (APS) should be classified as type I arylsulphatases. This type of enzyme is strongly inhibited by cyanide but is not appreciably affected by sulphate or phosphate. On the other hand, those enzymes which show relatively little affinity and activity towards NPS and APS but considerable affinity and activity towards the more complex arylsulphate, dipotassium 2-hydroxy-5-nitrophenyl sulphate (nitrocatechol sulphate, NCS), are classified as type II arylsulphatases. These enzymes are strongly inhibited by sulphate and phosphate but are unaffected by cyanide. Considerable information on the mode of action of one example of a type I enzyme (the bacterial arylsulphatase from *Alcaligenes metalcaligenes*) has already been obtained (Dodgson, Spencer & Williams, 1955, 1956*a*).

Examples of both types of arylsulphatases occur in human liver and other tissues (Dodgson, Spencer & Wynn, 1956*b*). Arylsulphatase C, a type I enzyme, is associated with the microsomes of the liver cell and has not yet been obtained in soluble form. Arylsulphatases A and B are type II enzymes and can readily be obtained in soluble form. The present communication describes the purification and properties of human-liver arylsulphatase B and gives some indication of the nature of the ionizing groups which are involved in the enzymic reaction. A preliminary account of this work has already appeared (Wynn & Dodgson, 1957).

* Part 18: Dodgson & Lloyd (1957).

MATERIALS AND METHODS

Substrates. APS, NPS and potassium phenyl sulphate were prepared by the method of Burkhardt & Lapworth (1926), NCS by the method of Roy (1953) as modified by Dodgson & Spencer (1956*a*) and the monopotassium salts of 4-hydroxy-2-nitrophenyl and 4-hydroxy-3-nitrophenyl sulphates by the method of Smith (1951). The monopotassium salts of *o*-hydroxyphenyl sulphate (catechol monosulphate) and 4-chloro-2-hydroxyphenyl sulphate (4-chlorocatechol monosulphate) were prepared from catechol and 4-chlorocatechol respectively by the method of Burkhardt & Lapworth (1926), but sufficient chlorosulphonic acid was used to sulphate one hydroxyl group only (cf. Dodgson, Rose & Spencer, 1955). Tyrosine *O*-sulphate was prepared by the method of Tallan, Bella, Stein & Moore (1955).

Buffers. In most of the enzyme experiments sodium acetate–acetic acid mixtures (hereafter referred to as acetate buffers) were used to control pH. However, in certain experiments where the properties of the human enzyme were being compared with those of the corresponding enzyme of ox liver, substrate (NCS) solutions were buffered with the acetate–HCl mixture (hereafter referred to as acetate–HCl buffer) described by Roy (1953, 1954). In preparing these solutions Roy used HCl to overcome the strong buffering action of the substrate, but when substrate solutions of varying concentration were required it automatically followed that different amounts of HCl had to be introduced in order to achieve the same pH.

Determination of enzyme activity. Enzyme activity towards APS, NPS and NCS was measured by spectrophotometric estimation of the respective liberated phenols (Dodgson, Spencer & Thomas, 1955). A similar method was

used to determine activity towards 4-hydroxy-2-nitrophenyl and 4-hydroxy-3-nitrophenyl sulphates, the anionic forms of the liberated phenols being estimated spectrophotometrically at a wavelength of 540 mμ. Enzyme activity towards all other substrates was determined by estimating liberated sulphate by the method of Dodgson & Spencer (1953). Except where otherwise stated, incubation of enzyme and substrate was for 1 hr. at 37·5° in the presence of 0·5 M-acetate buffer adjusted to the appropriate pH. One unit of arylsulphatase B activity is defined as that which liberates 1 μg. of nitrocatechol in 1 hr. at 37·5° from 0·01 M-NCS in the presence of 0·5 M-acetate buffer, pH 6·1.

Determination of protein. The method of Lowry, Rosebrough, Farr & Randall (1951) was used. A calibration curve was constructed with varying concentrations of the stage 3 enzyme preparation and spectrophotometric readings were made at a wavelength of 750 mμ.

Determination of nucleic acid. The ratio of the spectrophotometric readings at 280 and 260 mμ was used to determine nucleic acid (Warburg & Christian, 1941).

EXPERIMENTAL AND RESULTS

Preliminary purification of arylsulphatase B

Post-mortem samples of non-pathological human livers were obtained within 48 hr. after death. Acetone-dried powders of these livers were made according to the directions of Dodgson, Rose, Spencer & Thomas (1957).

Stage 1. Acetone-dried liver (60 g.) was suspended (Townson & Mercer top-drive macerater) in 500 ml. of 0·1 M-sodium acetate solution which had been adjusted to pH 7 with a few drops of acetic acid. After incubating at 37·5° for 30 min. the suspension was cooled to 0° and clarified by centrifuging at 6000 *g* and 0° for 30 min. The supernatant was kept at 0° whilst the debris was washed by resuspending it in a further 300 ml. of the acetate mixture and centrifuging as before. The two supernatants (approx. 700 ml.) were combined and diluted with sufficient water (usually about 300 ml.) to give a final protein concentration of 0·5 %.

Stage 2. The enzyme solution was cooled to 0° and acetone (at 0°) was added slowly with stirring until the concentration of acetone was 40 % (v/v). The whole was allowed to stand at −5° for 20 min. and the precipitate was separated by centrifuging at −5° and suspended in a volume of water equal to the volume of acetone originally added. After dialysing overnight against running water the suspension was clarified by centrifuging at 25 000 *g* and 0° for 30 min., and the clear supernatant solution diluted with water to give a final protein concentration of 0·25 %.

Up to this stage in the purification procedure it was not possible to determine the activity of arylsulphatase B with certainty owing to the presence of arylsulphatase A in the enzyme preparation. However, most of the latter enzyme is eliminated during stage 2 (cf. Dodgson *et al.* 1956*b*). The arylsulphatase B activity of the final solution was approx. 200 units/mg. of protein.

Stage 3. Sufficient sodium acetate and $CaCl_2$ were added to the enzyme solution to give final concentrations of 0·03 and 0·003 M respectively and the pH was adjusted to 6·5 with a few drops of acetic acid. After cooling to 0° the solution was treated with acetone at 0° until the concentration of acetone was 30 % (v/v), and after standing at −5° for 30 min. the precipitate was separated by centrifuging at 6000 *g* and −5° for 20 min. The precipitate was dissolved in water (150 ml.) before dialysing for 24 hr. against running water. The cloudy solution was clarified by centrifuging at 25 000 *g* and 0° for 30 min. The arylsulphatase B activity of the final clear solution was approx. 400 units/mg. of protein and the total yield of enzyme was 60 % of that present at stage 2.

Stage 4. The enzyme solution (approx. 175 ml.) was dialysed at room temperature for 3 days against several changes of distilled water (a total of 15 l./50 ml. of enzyme solution). The precipitate which appeared was separated by centrifuging at 25 000 *g* and 0° for 30 min. The bulk of the enzyme activity was present in this precipitate which was then allowed to stand in the presence of M-sodium acetate (20 ml./50 ml. of the stage 3 preparation) at 0° for 24 hr. This treatment dissolved most of the arylsulphatase B, the final enzyme solution being clarified by centrifuging as before. The arylsulphatase B activity of this preparation was approx. 800 units/mg. of protein; yield, 40 % of stage 3.

Properties of the stage 4 *enzyme.* Typical substrate concentration–activity and pH–activity curves for the stage 4 enzyme are shown in Figs. 1 and 2 respectively. Both curves exhibited marked anomalies which varied from preparation to preparation. Further investigations showed that enzyme activity could be markedly affected by varying the concentration of acetate buffer in the incubation mixture (Fig. 3). It seemed possible that the presence of small amounts of arylsulphatase A in the preparation might be responsible for these anomalies, but paper electrophoresis of the preparation under the conditions described by Dodgson & Spencer (1956*b*) gave no evidence of the presence of this enzyme. Moreover, Dodgson & Spencer (1956*a*, *c*) have shown that human arylsulphatase A gives an anomalous time–activity curve when assayed against 0·005 M-NCS in the presence of 0·5 M-acetate buffer, pH 5. The time–activity curve of the stage 4 enzyme preparation, when measured under these conditions, was linear and showed no anomalies. It was therefore tentatively assumed that the anomalies shown by arylsulphatase B were due to the varying tendency of the enzyme protein to combine with inert

protein under the varying conditions of the enzyme experiments and attempts were therefore made to purify the enzyme further.

Further purification of the enzyme

Stage 5. The stage 3 enzyme preparation was used as starting material for further purification.

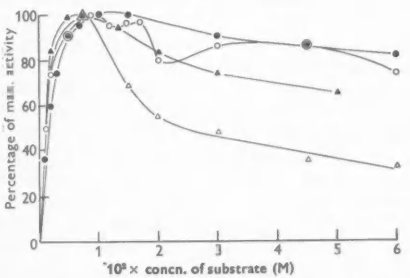

Fig. 1. Substrate concentration–activity curves for human arylsulphatase B acting on NCS. Incubation was for 1 hr. at 37·5°. ○, Stage 4 enzyme incubated in the presence of 0·5 M-acetate buffer, pH 6·1; ●, stage 6 enzyme in the same buffer; △, stage 4 enzyme or stage 6 enzyme incubated with substrate solutions buffered at pH 5·7 with acetate–HCl according to the directions of Roy (1954); ▲, stage 6 enzyme incubated in the presence of 0·5 M-acetate buffer, pH 5·7. The curves are not quantitatively related to each other.

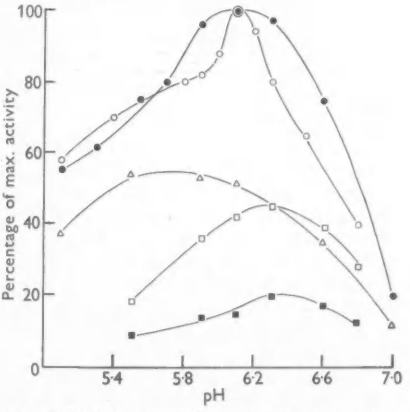

Fig. 2. pH–Activity curves for arylsulphatase B of human liver. The final concentration of substrate (NCS) was 0·01 M and incubation was for 1 hr. at 37·5° in the presence of 0·5 M-acetate buffer. ○, Stage 4 enzyme; ●, stage 6 enzyme; ■, stage 6 enzyme in the presence of 0·0025 M-NaH_2PO_4; □, stage 6 enzyme in the presence of 0·1 M-Na_2SO_4; △, stage 6 enzyme in the presence of 0·08 M-KCl. Stage 6 enzyme curves are quantitatively related to each other.

The enzyme solution (usually in 80 ml. portions) was adjusted to pH 7 and an aqueous solution of protamine sulphate (0·1 %), which had been adjusted to pH 8 with N-NaOH, was added slowly until the clear supernatant obtained by centrifuging at 25 000 *g* and 0° for 5 min. no longer gave a precipitate on adding further protamine sulphate solution. The final clear supernatant was adjusted to pH 9 with N-NaOH, the precipitate removed by centrifuging as before and the supernatant was dialysed for 12 hr. at 5° against distilled water (100 vol.). After clarifying by centrifuging, the arylsulphatase B activity of the solution was approx. 1000 units/mg. of protein. Yield, 90 % of stage 3. The concentration of nucleic acid in the enzyme preparation was reduced from approx. 0·8 % to zero during this procedure.

Stage 6. Sufficient sodium acetate was added to the enzyme solution to give a final concentration of 0·1 M and the whole was adjusted to pH 6 with acetic acid. This solution was allowed to filter by gravity at room temperature through dry grade 4 sintered-glass funnels (no more than 1000 units of activity/funnel) which had previously been cleaned with boiling nitric acid followed by boiling water. The diameter of the sintered-glass disk of each funnel was approx. 30 mm. An appreciable amount of inert protein passed through the sintered disks which, however, retained a considerable part of the arylsulphatase activity. Each sintered disk was washed (by gravity) with 2 ml. of 0·1 M-acetate buffer, pH 6, and the enzyme was subsequently eluted from the disk with 3 ml. of M-acetate buffer, pH 6. The arylsulphatase B activity of the final

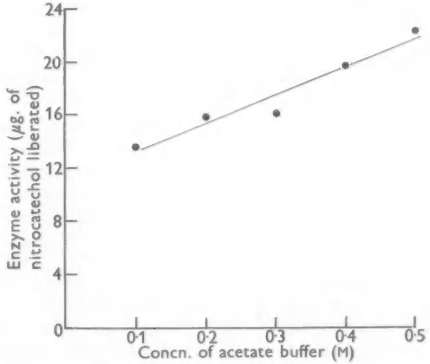

Fig. 3. Effect of buffer concentration on the activity of human arylsulphatase B at stage 4 in the purification procedure. The final concentration of substrate was 0·01 M-NCS and incubation was for 1 hr. at 37·5° in the presence of varying concentrations of acetate buffer, pH 6·1.

solution was approx. 3000 units/mg. of protein. Yield, 40 % of stage 5. Examination of the various filtrates and the original enzyme solution by phase-contrast microscopy suggested that true adsorption of enzyme on glass rather than a simple filtration of particulate enzyme was involved. This was confirmed by the finding that the enzyme was also strongly adsorbed on powdered glass and on Ballotini no. 12 glass beads (English Glass Co. Ltd., Leicester).

Properties of the purified enzyme

Substrate concentration–activity and pH–activity curves for the purified enzyme are shown in Figs. 1 and 2 respectively. The curves no longer exhibited the anomalies shown by the stage 4 enzyme preparation, although the pH–activity curve was still asymmetric. Acetate concentration no longer affected the activity of the enzyme over the concentration range studied (0·1–1 M).

The behaviour of the enzyme towards increasing concentrations of substrate (optimum substrate concentration of 0·01 M-NCS in 0·5 M-acetate buffer, pH 6·1) contrasts sharply with that of the partially purified corresponding enzyme of ox liver. With the latter Roy (1954) observed that, at the optimum pH (5·7), arylsulphatase activity continued to increase with increasing substrate concentration and failed to reach a maximum even at the practical limit of substrate solubility (0·06 M-NCS). However, as mentioned earlier, the method used by Roy (1954) to prepare substrate solutions was such that an increase in the concentration of NCS was paralleled by an increase in the concentration of Cl^- ions. Exact repetition of Roy's work with the ox enzyme has confirmed his observations (see Fig. 4). However, when Cl^- ions were omitted from the incubation mixtures a considerable change in the substrate concentration–activity curve was obtained. Moreover, the shape of the curve was markedly affected by the concentration of the acetate buffer used (Fig. 4). Since activity of the human enzyme was also affected by acetate concentration during the early stages of purification, the ox enzyme was purified further (× 15) by treatment with protamine sulphate and adsorption on sintered-glass disks as described earlier for the human enzyme. Although the activities of the two enzymes/mg. of protein were now approximately comparable the ox enzyme was still affected by acetate concentration and the substrate concentration–activity curve, when determined in the presence of increasing concentrations of Cl^- ions at pH 5·7, was similar to that obtained before further purification. Under identical experimental conditions (i.e. pH 5·7 in acetate–HCl buffer) both the stage 4 and stage 6 preparations of the human enzyme gave substrate concentration–activity curves which deviated from the normal curve obtained at this pH only by virtue of an apparent increase in the degree of inhibition by excess of substrate (see Fig. 1). However, this increase can probably be attributed to the inhibiting effect of Cl^- ions, substrate solutions of higher concentration containing greater amounts of these ions. Fig. 2 shows the effect of Cl^- ions (0·08 M-KCl) on the pH–activity curve of the human enzyme. Considerable inhibition occurs under these conditions and there is a shift in the pH optimum in the direction of lower pH. Roy (1955) has observed a similar shift in the pH optimum of the ox enzyme when determined in the presence of 0·08 M-KCl, but this was accompanied by an appreciable increase in the activity of the enzyme. It is possible therefore that the anomalous substrate concentration–activity curve obtained for the ox enzyme when acetate–HCl buffer is used is at least partly due to the activating effect of the increasing amounts of Cl^- ions which parallel the increases in substrate concentration.

In certain other respects the two enzymes exhibit similar behaviour. Fig. 2 shows the effect of $H_2PO_4^-$ and SO_4^{2-} on the pH–activity curve of the stage 6 human enzyme. Considerable inhibition occurs with both ions, the resultant curves being very similar to those obtained by Roy (1955) for the ox enzyme.

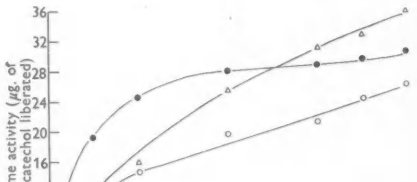

Fig. 4. Substrate concentration–activity curves for the arylsulphatase B of ox liver acting on NCS. Incubation was for 1 hr. at 37·5°. ○, Ox arylsulphatase B prepared according to the directions of Roy (1954) and incubated in the presence of 0·1 M-acetate buffer, pH 5·7; ●, the same concentration of enzyme incubated in the presence of 0·5 M-acetate buffer, pH 5·7; △, the same concentration of enzyme incubated with substrate solutions buffered at pH 5·7 with acetate–HCl according to the directions of Roy (1954), and which therefore contained varying amounts of Cl^- ions.

Nature of the dissociating groups involved in the enzyme reaction

In the experiments described below, Michaelis constants (K_m) and maximum velocities

$$(V_{max.} = k_3[E])$$

were determined by the method of Lineweaver & Burk (1934), with case III, which takes into account any inhibition by excess of substrate. Values of $V_{max.}$ are relative values expressed in arbitrary units since, although the concentration of enzyme used was the same in all experiments, the absolute concentration [E] was unknown.

Variation of K_m with pH. A number of workers have shown that information regarding the functional groups of enzymes can be obtained by consideration of the variation of the dissociation constant (K_s) of the enzyme–substrate complex with pH (see Bray & White, 1957), and Dixon (1953) has formulated certain rules which govern the interpretation of these variations. Under certain circumstances when, as in the present case, direct determination of K_s is not possible, these rules can be applied to the variation of the Michaelis constant (K_m) with pH. These circumstances have previously been discussed by Dodgson *et al.* (1955) and will not therefore be reiterated. It suffices to say that Dixon (1953) states that rules 9 and 10 can be applied to the pK_m (p$K_m = -\log K_m$)–pH curve in those regions where the pV

$$(pV = -\log V_{max.})\text{–pH}$$

curve is either (1) horizontal or (2) has non-whole-number slopes or is curved, provided that, in the second case, the pK_m–pH curve has whole-number slopes (e.g. 0- or 1-unit slopes) in the same pH regions.

Fig. 5 shows the pK_m–pH and pV–pH curves for arylsulphatase B acting on NCS and it may be deduced from these curves that Dixon's rules are applicable in the present case. It can be seen from Fig. 5 that there is a change in direction of the pK_m–pH curve in the region of pH 5·7. According to Dixon's rule 10 this change in direction reflects the pK value of an ionizing substrate grouping or a substrate-binding enzyme grouping. The substrate contains no ionizing group with pK in the region of 5·7 (the pK of the free phenolic group of NCS is 6·5; Dodgson *et al.* 1955) and the change in direction at this pH may therefore be presumed to be due to the ionization of a substrate-binding enzyme group.

Further information may be derived from the pK_m–pH curve by considering Dixon's rule 9, which states that the slope in any region of the curve is numerically equal to the change of charge on...'desubstration', that is to say, it is equal to the number of charges on the left-hand side of the equation

$$E + S \rightleftharpoons ES \qquad (1)$$

minus the number of charges on the right-hand side, due regard being paid to algebraic sign. Since there is no evidence from the pK_m–pH curve of change in charge of enzyme–substrate complex or of substrate over the pH range studied it follows that the enzyme group with pK 5·7 is of a type which gains a negative charge or loses a positive charge above pH 5·7 to give a -1 unit slope.

Variation of $V_{max.}$ with pH. Fig. 6 shows the variation of the maximum velocity ($V_{max.} = k_3[E]$) with pH. From Figs. 5 and 6 it can be seen that whereas, over the range pH 5–5·7, K_m remained constant there was a significant increase in $V_{max.}$.

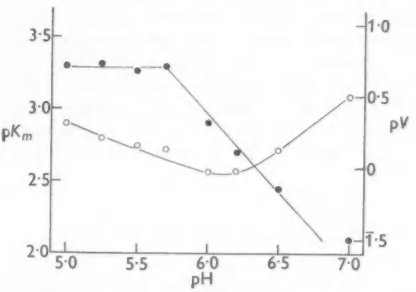

Fig. 5. Variation with pH of pK_m ($=\log K_m$) and pV ($=\log V_{max.}$) for human arylsulphatase B acting on NCS. Incubation was for 1 hr. at 37·5° in the presence of 0·5 M-acetate buffer. ●, pK_m; ○, pV.

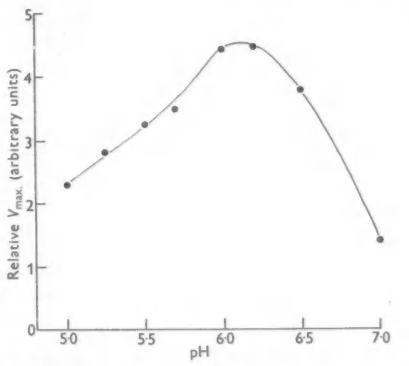

Fig. 6. Variation with pH of the maximum velocity ($V_{max.}$) of the hydrolysis of NCS by human arylsulphatase B. Incubation was for 1 hr. at 37·5° in the presence of 0·5 M-acetate buffer. The values of $V_{max.}$ are relative values expressed in arbitrary units.

Similar findings have been made for uricase (Baum, Hübscher & Mahler, 1956) and for chymotrypsin acting on acetyl-L-phenylalanine ethyl ester (Hammond & Gutfreund, 1955). It must be assumed that the reaction at the specific substrate-binding site of the enzyme, resulting in the formation of the enzyme–substrate complex, is independent of the subsequent reaction of the substrate with the catalytic site(s) of the enzyme.

It has been pointed out (Botts & Morales, 1953) that changes in $V_{max.}$ which occur on the acid side of the pH optimum of an enzyme can be attributed to non-competitive inhibition of a catalytic site of the enzyme by H^+ ions. In such a case the ionization constant of the catalytic site is synonymous with the inhibitor constant, $K_i=[E]\,[H^+]/[EH^+]$, where $[E]$ is the concentration of active enzyme and $[EH^+]$ is the concentration of inhibited enzyme. Hammond & Gutfreund (1955) have pointed out that, from the equations given by Lineweaver & Burk (1934), the following relationship can be derived between inhibitor concentration $[H^+]$, $k_3[E]$ (the maximum velocity at a particular concentration of $[H^+]$) and $k_3[E_0]$ (the maximum velocity when all the enzyme is present in the active form):

$$[E_0]/[E]=1+[H^+]/K_i. \qquad (2)$$

K_i may be calculated from the slope $(1/K_i)$ of the curve obtained by plotting $[E_0]/[E]$ against $[H^+]$. Fig. 7 shows the plot of $[E_0]/[E]$ against $[H^+]$ for arylsulphatase B, the ratio $[E_0]/[E]$ for the different values of $[H^+]$ being calculated from determinations of $k_3[E]$, assuming $k_3[E_0]=k_3[E]$ at pH 6·1. The value for K_i (and hence the ionization constant of the catalytic site) obtained from this graph is 7×10^{-6} and pK_i $(-\log K_i)=5{\cdot}2$.

Further information regarding the nature of the catalytic sites present in the enzyme–substrate complex can be obtained from the $V_{max.}$–pH curve (Fig. 6). Michaelis & Davidsohn (1911) and Michaelis & Pechstein (1914) suggested that the bell-shaped curve which is generally obtained when $V_{max.}$ is plotted against pH is the result of the ionization of acidic and basic groups of the enzyme. On the basis of this concept, Alberty & Massey (1954) suggested that the reaction mechanism (3) could apply to such a system.

(3)

In reaction (3) EH is the catalytically active form of the enzyme and K_a and K_b are acid dissociation constants for the two groups. By steady-state treatment these workers derived the following expression for the maximum velocity:

$$V_{max.}=\frac{k_3[E_0]}{1+[H]/K'_a+K'_b/[H]}. \qquad (4)$$

If the symbol $V_{(max.)}$ be allowed to represent the highest maximum velocity obtained at a H^+ ion concentration $H_{max.}$ (i.e. the highest point on the $V_{max.}$–pH curve) then it is possible to derive from this expression the following equations:

$$K'_aK'_b=(H_{max.})^2, \qquad (5)$$

$$K'_a=H_a+H_b-4H_{max.}, \qquad (6)$$

where H_a is the H^+ ion concentration at which the value $V_{max.}/V_{(max.)}$ on the acid side of $H_{max.}$ is equal to 1/2 and H_b is the H^+ ion concentration at the corresponding point on the basic side (Alberty & Massey, 1954). K'_a and K'_b for arylsulphatase B were 5·3 and 6·9 respectively. The value 5·3 is in good agreement with the value (5·2) obtained by the method of Hammond & Gutfreund (1955). To summarize, there are present in the enzyme–substrate complex two ionizing groups with pK in the region of 5·3 and 6·9 respectively, the ionization of which affects the breakdown of the enzyme–substrate complex.

Asymmetric nature of the $V_{max.}$–pH *curve.* It will be noted from Fig. 6 that the $V_{max.}$–pH curve decreases more sharply on the basic side of the maximum than on the acidic side. Alberty & Massey (1954) have pointed out that such asymmetric curves could occur if EH_2S or ES (see eqn. 3) or both were also able to break down to yield product, though at slower rates than EHS. They argue that the fact that zero activity is generally

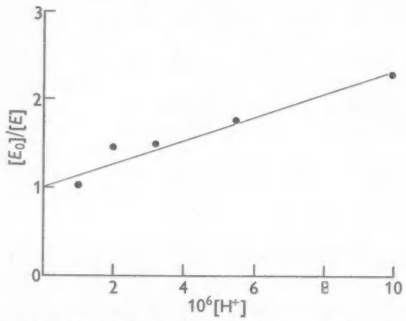

Fig. 7. Effect of H^+-ion concentration on the maximum velocity of the hydrolysis of NCS by human arylsulphatase B. The curve is plotted according to the method of Hammond & Gutfreund, 1955 (see text).

approached in solutions of increasing acidity or basicity indicates that such reactions may be ignored. However, the reaction mechanism proposed by these workers (eqn. 3) assumes that the substrate remains in the same state of ionization throughout the pH range studied. In the case of arylsulphatase B the free phenolic group of the substrate (NCS) is known to dissociate (pK 6·5) and in such a case it becomes necessary to consider a system in which the substrate, in addition to the enzyme, can exist in more than one ionic form. Laidler (1955*a*, *b*) has considered systems of this type from a theoretical point of view and has pointed out that ionization of substrate could affect the symmetry of their $V_{max.}$–pH curves. In the present case there is no reason to suppose that the pK of the free phenolic group of the substrate is appreciably altered when present in the enzyme–substrate complex, and it seems possible that increasing ionization of this group on the basic side of the $V_{max.}$–pH curve could be responsible for the sharper decline in enzyme activity which occurs on this side of the curve.

Activity of the enzyme towards other arylsulphates

The activity of the enzyme towards certain other arylsulphates was tested under arbitrary experimental conditions. The results are shown in Table 1, from which it can be seen that, under the conditions used, the enzyme showed little activity towards potassium phenyl sulphate and its monosubstituted derivatives. On the other hand, appreciable activity towards the disubstituted derivatives was obtained. A systematic study is now being made in order to establish more clearly the effect of molecular configuration of substrate on enzyme activity.

Table 1. *Relative activity of human arylsulphatase B towards the potassium salts of certain arylsulphates*

The stage 6 enzyme preparation was used and the final concentration of each substrate was 0·0125M in 0·5M-acetate buffer, pH 6·1. Incubation was for 1 hr. at 37·5°.

Arylsulphate	Arylsulphatase activity (μg. of SO_4^{2-} ion liberated/hr.)
2-Hydroxy-5-nitrophenyl sulphate	30
4-Hydroxy-3-nitrophenyl sulphate	34
4-Hydroxy-2-nitrophenyl sulphate	5
4-Chloro-2-hydroxyphenyl sulphate	10
o-Hydroxyphenyl sulphate	0
Phenyl sulphate	0
p-Acetylphenyl sulphate	0
p-Nitrophenyl sulphate	2
Tyrosine *O*-sulphate	0

DISCUSSION

The purification procedure described for human arylsulphatase B has been successfully applied to a large number of different samples of human liver. It should be noted, however, that it is important to test the liver samples for arylsulphatase activity towards NCS before purification since occasionally livers have been obtained which possessed negligible activity. On two other occasions the purification procedure has failed to work satisfactorily. No attempts have been made to investigate these points fully but the explanation probably lies in the variation in the degree of degenerative change occurring in the liver between the times of death and the post-mortem sampling.

The reasons for the differences observed between the ox and human enzymes are not yet clear. They may be fundamental in character or they may merely reflect degenerative changes which may have taken place in the human liver and which would be minimal in the fresh ox-liver samples. Such changes might result in modification of the enzyme or of other protein constituents of the liver with which the enzyme is associated. In this connexion it is worth noting that, irrespective of the degree of purification, the human-liver enzyme has never given an anomalous substrate concentration–activity curve corresponding to that obtained for the ox enzyme (see also Dodgson *et al.* 1956*b*). On the other hand, Dodgson & Spencer (1957*b*) obtained a substrate concentration–activity curve for the arylsulphatase activity of human serum which was still ascending at a concentration of 0·06M-NCS and which closely resembled that obtained for the ox-liver enzyme buffered with acetate–HCl.

The investigations on the mechanism of action of human arylsulphatase B have indicated that there is present in the enzyme an ionizing group with pK about 5·7 which is at least partly responsible for the binding of substrate. The group loses a positive charge (or gains a negative charge) above pH 5·7 but its chemical nature is at present obscure. The reaction of substrate with this group, resulting in the formation of the enzyme–substrate complex, is apparently independent of the subsequent reaction of the substrate with the catalytic site(s) of the enzyme. Not only does the maximum velocity of the enzyme reaction increase appreciably over the pH range (5·0–5·7) where the affinity of enzyme for substrate remains constant but it continues to increase between pH 5·7 and 6·1 where enzyme–substrate affinity is decreasing sharply.

It has also been shown that there are present in the enzyme–substrate complex two groups, the ionizations of which affect the breakdown of the

complex. The pK of one of these groups has been determined by two different methods as being about 5·2. It is questionable, however, whether the pK 6·9 obtained for the second group can be regarded as significant in view of the asymmetric nature of the $V_{max.}$–pH curve from which it was calculated. If ionization of the free phenolic group of the substrate is responsible for the fact that this curve decreases more sharply on the basic side of the maximum than on the acid side then the value pK 6·9 calculated for the basic catalytic site of the enzyme will be a composite value of the pK values of the phenolic substrate group and the basic catalytic site when both are present in the form of enzyme–substrate complex.

It is already clear that the mechanism of action of human arylsulphatase B (a type II arylsulphatase) differs in one respect from that of the arylsulphatase of *Alcaligenes metalcaligenes* (a type I enzyme). With the latter enzyme no indications were obtained that the binding of substrate by the enzyme was independent of the subsequent reaction of substrate with the catalytic site(s) of the enzyme since there was no significant change in $V_{max.}$ over the pH range where K_m remained constant (see Dodgson *et al.* 1955). It will now be important to examine other examples of both types of arylsulphatase in order to establish whether difference in reaction mechanism is the fundamental reason for the differences observed in the properties of the two types of enzyme.

The meagre evidence available at present suggests that the activity of arylsulphatase B is greatly enhanced by the presence in the substrate of a free phenolic group in addition to a second substituent group. In the absence of this free phenolic substrate group the enzyme is only feebly active towards arylsulphates. If ionization of the free phenolic group of NCS is a contributory factor to the sharp decrease in the $V_{max.}$–pH curve which occurs on the basic side of the maximum, the further implication is that maximum activity of the enzyme is also dependent on this free phenolic group being present in an unionized form. Further experiments are now being conducted in these Laboratories in order to test the validity of these various possibilities.

SUMMARY

1. Human-liver arylsulphatase B has been considerably purified by a procedure involving acetone-fractionation, treatment with protamine sulphate and adsorption of the enzyme on sintered-glass disks.

2. During the early stages of the procedure the enzyme was markedly affected by buffer concentration and gave anomalous substrate concentration–activity curves. These effects were not obtained with the final enzyme preparation. Comparatively crude preparations of the corresponding ox-liver enzyme exhibited similar anomalies which, however, were not removed by further purification.

3. With dipotassium 2-hydroxy-5-nitrophenyl sulphate as assay substrate some indications of the nature of the ionizing groups which are involved in the formation and subsequent breakdown of the enzyme–substrate complex have been obtained from a study of the variation of K_m and $V_{max.}$ with pH.

4. The enzyme showed little activity towards potassium phenyl sulphate and its monosubstituted derivatives; there was appreciable activity towards disubstituted derivatives.

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Biological Synthesis of Ascorbic Acid: L-Galactono-γ-lactone Dehydrogenase

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The terminal step in the biosynthesis of L-ascorbic acid in peas has been shown to be the oxidation of L-galactono-γ-lactone by an enzyme present in mitochondria (Mapson, Isherwood & Chen, 1954). This enzyme has been solubilized from mitochondria obtained from freshly germinated peas, cabbage leaves and cauliflower florets. Since the enzyme preparations from the mitochondria of the cauliflower florets were found to be more active than those from either peas or cabbage, we have used this material in most of the work reported here. The present paper describes the preparation and partial purification of the enzyme, together with an account of some of its properties.

EXPERIMENTAL

Chemicals

L-Galactono-γ-lactone was prepared by the reduction of D-galacturonic acid by borohydride as follows. D-Galacturonic acid (10 g.) was dissolved in 40 ml. of water and neutralized with NaOH to between pH 8·5 and 9·0. Borohydride was added gradually with stirring at room temperature. Samples were removed and acidified with acetic acid to remove excess of borohydride, and galacturonic acid was tested for by boiling with Fehling's solution. After reduction was complete, the solution was acidified with acetic acid to pH 5·0, a slight excess of barium acetate added, and the precipitate filtered off. Ethanol (2 vol.) was added to the solution and the precipitate was collected. After the precipitate had been washed twice with 60% (v/v) ethanol, barium was removed by Dowex 50 resin, and to the filtrate 1–2 drops of 3N-HCl were added. The solution was concentrated to a syrup and dried *in vacuo*. The lactone was recrystallized from absolute ethanol.

L-Gulono-, D-altrono-, D-glucono- and D-mannono-γ-lactone were prepared as described by Isherwood, Chen & Mapson (1954). Glucurono-γ-lactone, D-gulono-γ-lactone and D-galactono-γ-lactone were purchased from L. Light and Co., Colnbrook, Bucks. Cytochrome *c* was prepared from ox heart by the method of Keilin & Hartree (1937).

Phenazine methosulphate was prepared by the addition of dimethyl sulphate to phenazine dissolved in nitrobenzene (Kehrmann, 1913; Hillemann, 1938). Flavin mononucleotide (FMN) and flavinadenine dinucleotide (FAD) were purchased from the Sigma Chemical Co., U.S.A.

Preparation of the enzyme

Extraction from mitochondria step 1. The florets (1 kg.) of cauliflower after separation from leaves and stalk were ground in a mechanical mortar with 1 l. of a sucrose–phosphate solution (0·4M-sucrose and 0·1M-Na phosphate buffer, pH 7·4). The mortar was cooled to −20° and the sucrose phosphate solution to 0° before grinding, which was carried out as rapidly as possible (usually within 30 min.) at room temperature. The mixture was pressed through muslin and centrifuged at 20 000 *g* for 20 min. The spun-down mitochondria were washed with 0·1M-phosphate buffer (pH 7·4), recentrifuged at 20 000 *g* for 20 min., and resuspended in 50–100 ml. of the same buffer. To this suspension was slowly added 10 times the volume of acetone, previously cooled to −20°. After 15 min. the precipitate was collected, the acetone partially removed *in vacuo*, and the residue suspended in 150 ml. of the phosphate buffer (0·1M). The suspension was dialysed against at least two changes of the same buffer for 3 hr. at +1° to remove most of the acetone.

Fractionation with ammonium sulphate step 2. The suspension was centrifuged to remove undissolved protein, and the clear solution brought to 40% saturation by the addition of 243 g. of $(NH_4)_2SO_4$/l. of solution. The precipitated protein was removed, and the solution brought to 70% saturation by the further addition of 205 g. of $(NH_4)_2SO_4$/l. The precipitate was collected, dissolved in 20 ml. of 0·01M-Na

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phosphate buffer (pH 6·8) and dialysed for 2 hr. against the same buffer at 1°.

Absorption and elution from calcium phosphate gel step 3. (At this stage and on all of the subsequent steps operations were carried out at a temperature of 1°.) The solution was treated with calcium phosphate gel (Keilin & Hartree, 1938) (1 mg. of gel/1·5 mg. of protein), left for 15 min. and centrifuged for 5 min. at 1500 *g*. The gel was washed with phosphate buffer 0·05 M (pH 6·8), recentrifuged, and the enzyme eluted with 0·5 M-Na phosphate buffer (pH 7·4). The eluate was dialysed against 0·01 M-phosphate buffer (pH 6·8) for 3 hr.

Second fractionation with ammonium sulphate step 4. The solution was brought to 53% saturation by the addition of 336 g. of $(NH_4)_2SO_4$/l. of solution, and after removal of the precipitated protein the solution was brought to 80% saturation [192 g. of $(NH_4)_2SO_4$/l.]; the precipitate was collected and dissolved in 5 ml. of 0·1 M-phosphate buffer (pH 6·8). The solution was dialysed for 2 hr. against the same buffer.

Absorption of contaminating protein with calcium phosphate gel step 5. The solution was treated with calcium phosphate gel at 1° (1 mg. of gel/1·5 mg. of protein), left for 15 min. and centrifuged. The gel was discarded and the solution containing the enzyme stored at −3°. This solution should contain not less than 10 mg. of protein/ml.; weaker solutions have been found to be unstable. The amount of enzyme lost and the purification achieved by these operations are summarized in Table 1.

Assay methods

Protein. The protein content of the various enzyme preparations was estimated by the method of Lowry, Rosebrough, Farr & Randall (1951).

Phenazine. The solubilized enzyme was unable to reduce indophenol, ferricyanide or tetrazolium at appreciable rates. It could, however, reduce phenazine methosulphate, and a spectrophotometric assay has been devised with this dye as hydrogen acceptor. The reaction mixture contained enzyme, L-galactono-γ-lactone, phenazine methosulphate and indophenol, the phenazine after reduction by the lactone in turn reducing 2:6-dichlorophenolindophenol, the course of this latter reduction being followed on an EEL colorimeter with an EEL 607 filter. The reaction was carried out anaerobically to prevent the reoxidation of the reduced phenazine. The Thunberg tubes used were specially constructed with a light-path of 1·2 cm. so as to fit the colorimeter. Because the product of the reaction is the reducing agent, ascorbic acid, 2 moles of indophenol are reduced for every mole of lactone oxidized.

The presence of an indophenol oxidase in the more impure preparations of the enzyme necessitated the use of cyanide during the assay. A blank rate must also be corrected for, the blank being determined by running all assay components with the exception of the enzyme. This blank is the result of some interaction between substrate, dyes and cyanide. With crude preparations of the enzyme, contaminating substances may be present which also reduce the dye. The true blank is then the blank as determined above plus the difference between the blank due to enzyme, cyanide and dye and that due to cyanide and dye alone.

In a Thunberg tube were placed the enzyme, 1 ml. of 0·01 M-KCN in 0·1 M-phosphate buffer (pH 7·4), 0·5 ml. of L-galactono-γ-lactone (24 mg./ml.) and phosphate buffer to a volume of 5 ml. In the side arm were placed 1 ml. of phenazine methosulphate (1 mg./ml.) in 0·1 M-phosphate buffer (pH 7·4) and 0·08 ml. of indophenol dye (1 mg./ml.). (The exact volume of indophenol may be varied slightly to give convenient optical-density values.) The tube was evacuated and flushed with N_2 several times and incubated for 10 min. at 37°. The dye was then tipped in and readings were taken on the colorimeter every 2 min. for 12 min.

Table 1. *Stages in the purification of* L-*galactono-γ-lactone dehydrogenase*

	Phenazine assay			Cytochrome assay			
Stage of purification	Total units	Recovery (% of original extract)	Specific activity (units/mg. of protein)	Total units	Recovery (% of original extract)	Specific activity (units/mg. of protein)	Cytochrome activity/phenazine activity
Phosphate (0·1 M) (pH 7·4) extract of acetone-dried powder (step 1)	985	—	2·7	8900	—	24	9
40–70% saturation $(NH_4)_2SO_4$ and dialysed (step 2)	630	64	4·4	7560	85	53	12
Treated with GSH and dialysed for 5 hr.	750	76	5·0	9680	109	65	13
Absorption on $Ca(PO_4)_3$ gel and elution (step 3)	515	53	13·2	5850	66	150	11
53–80% saturation $(NH_4)_2SO_4$ and dialysed (step 4)	168	17	12	2800	31	200	17
Treated with GSH and dialysed for 5 hr.	254	26	18	2910	33	208	12
Treated with $Ca(PO_4)_3$ gel in 0·1 M-phosphate (pH 7·4) (step 5)	161	16	16·5	2440	27	250	15
Supernatant treated with GSH and dialysed	270	27	27·5	2950	33	300	11

One unit of enzyme was defined as the amount that decreased the optical density by one scale unit/min. Proportionality between rate of reaction and enzyme concentration was achieved with an error of less than 10% over a tenfold range of enzyme concentration.

Cytochrome c. Reduction of cytochrome *c* was followed by measuring the increase in absorption at 550 mμ in a Hilger Uvispek spectrophotometer at room temperature. In a final volume of 3 ml. of 0·01 M-phosphate buffer (pH 7·8) were placed the enzyme, L-galactono-γ-lactone (2 mg./ml.) and cytochrome *c* (1·5 mg./ml.). Under these conditions the rate of reaction was proportional to enzyme concentration and was linear with time over periods of 15 min. The unit of enzyme activity was defined as that amount of enzyme which increased the optical density by 0·001/min. at room temperature (20°).

Formation, isolation and identification of the bis-2:4-dinitrophenylhydrazone of 2:3-dioxo-L-gulonic acid

Formation and isolation. L-Galactono-γ-lactone (2 mg./ml.) was dissolved in 4·5 ml. of 0·1 M-phosphate buffer (pH 7·4) to which phenazine (1 mg.) dissolved in 1 ml. and the necessary amount of enzyme in 0·5 ml. of the same buffer were added. After incubation at 35°, 1 ml. samples were removed and mixed with 4 ml. of 5% trichloroacetic acid. Precipitated protein was removed, if necessary, and the solution treated with the 2:4-dinitrophenylhydrazine reagent (Roe & Kuether, 1943). The formation and estimation of the phenylhydrazone was carried out in the manner described by these authors. The hydrazone was isolated from similar mixtures, after incubation with the phenylhydrazine reagent, by centrifuging and recrystallization from either acetic acid or acetone–methanol (1:1, v/v) solution. For comparison the bis-2:4-dinitrophenylhydrazone of 2:3-dioxo-L-gulonic acid was prepared from L-ascorbic acid, after oxidation with bromine, in a similar manner.

Identification. The hydrazones were analysed by paper chromatography with an aqueous solvent containing 10% (v/v) of acetic acid, 10% (v/v) glycerol and 10% (w/v) phenol; both hydrazones ran as a single spot with an R_F of 0·59. By using the upper phase of a mixture composed of *tert.*-amyl alcohol–ethanol–water (50:5:40, by vol.) and developing the chromatogram on paper previously buffered with 0·2 M-phosphate (pH 6·25), a similar result was obtained. The hydrazones ran as single spots with an R_F of 0·86.

The absorption spectra of both hydrazones were identical, with absorption maxima at 370 and 530 mμ, and the ratios of the values of the optical densities measured at 530 and 450, 370 and 450, and 370 and 530 for the enzymic and synthetically produced hydrazones were 2·02, 2·25 and 1·11 and 2·05, 2·29 and 1·05 respectively. These values agreed well with those reported by Probst & Schultze (1950) for the same compound.

RESULTS

Electron acceptors

As is shown below, in the absence of a suitable electron acceptor there was no oxidation of L-galactono-γ-lactone by the dehydrogenase. Various substances were tested for their ability to act as electron acceptors with the solubilized enzyme; these included methylene blue, ferricyanide, tetrazolium, 2:6-dichlorophenolindophenol, phenazine methosulphate and cytochrome *c*. Only the last two were reduced by the enzyme in the presence of substrate at any significant rate. Reduction of cytochrome *c* may be followed either aerobically or anaerobically since it proceeds equally well under either condition. Reduction of phenazine is more conveniently carried out under anaerobic conditions, so as to avoid the autoxidation of phenazine which would interfere with the reduction of indophenol.

Product of reaction

Ascorbic acid is oxidized by both phenazine and cytochrome c; so with both these substances as electron acceptors the ascorbic acid formed in the enzyme reaction is further oxidized under the conditions of the test to dehydroascorbic acid, which at the pH of the reaction mixture is in turn rapidly converted into 2:3-dioxo-L-gulonic acid (Penney & Zilva, 1943). The formation of ascorbic acid has therefore been shown to have occurred by

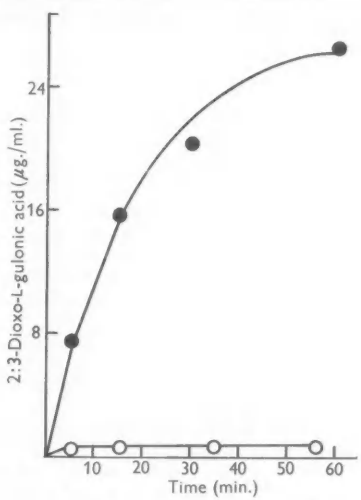

Fig. 1. Formation of 2:3-dioxo-L-gulonic acid by the enzymic oxidation of L-galactono-γ-lactone with phenazine as electron acceptor. Each solution in a total vol. of 6 ml. contained 4·5 ml. of 0·1 M-phosphate buffer (pH 7·4), 6 mg. of L-galactono-γ-lactone, 1 mg. of phenazine methosulphate. To one solution (●) 0·1 ml. of enzyme preparation (specific activity 250) was added, the other (○) serving as control. 1 ml. of reaction mixture was removed at intervals for the estimation of 2:3-dioxo-L-gulonic acid; temp. 37°.

the isolation and identification of the bis-2:4-dinitrophenylhydrazone of 2:3-dioxo-L-gulonic acid. The derivative prepared experimentally by the enzymic oxidation of L-galactono-γ-lactone was shown to be identical with that prepared from L-ascorbic acid after oxidation by bromine by (1) their similar behaviour when chromatographically analysed, (2) their identical absorption spectra and (3) the close agreement between the ratios of the optical densities measured at wavelengths of 530 and 450, 370 and 450, and 370 and 530 mμ; the last property has been shown to be an excellent criterion of identity and purity of this compound (Probst & Schultze, 1950).

The formation of 2:3-dioxo-L-gulonic acid during the course of the enzymic oxidation of L-galactono-γ-lactone with phenazine (Fig. 1) has been followed by preparing the 2:4-dinitrophenylhydrazone derivative according to the method of Roe & Kuether (1943). Similar results have been obtained with cytochrome *c*; in neither experiment was 2:3-dioxo-L-gulonic acid formed in the absence of an electron acceptor. This procedure as a method of assay is not as satisfactory as either of the methods in which the reduction of phenazine or cytochrome *c* is followed, owing to the instability of 2:3-dioxo-L-gulonic acid at the pH required for enzyme action (Penney & Zilva, 1943). Borate, which stabilizes 2:3-dioxo-L-gulonic acid, cannot be used, since it also forms complexes with L-galactono-γ-lactone.

Properties

Sulphydryl requirement. The enzyme appears to require sulphydryl groups for its activity, for this was inhibited, in both the phenazine and cytochrome *c* reactions, by reagents which inactivate these groups. *o*-Iodosobenzoate, Cu^{2+} ions and *p*-chloromercuribenzoate (PCMB) all inhibited strongly (50% or more) at concentrations of 0·1 mM. Iodobenzoate, oxidized glutathione and *p*-aminophenyl arsenoxide did not inhibit at a level of 0·1 mM even after 30 min. preincubation with the enzyme. The inhibition by PCMB has been studied in more detail and the results (Table 2) show that inhibition by the mercurial was immediate and was not noticeably increased when the enzyme was incubated with the inhibitor for 30 min. at 0°, although with longer periods of incubation total inactivation was observed. Complete reversal of this inhibition occurred if reduced

Table 2. *Inhibition by* p*-chloromercuribenzoate and reactivation by glutathione*

Expt.	Treatment	Reduction (% of control) Phenazine	Cytochrome *c*
1	Enzyme treated with PCMB (0·4 mM) for 30 min. at 0° before test	0	—
2	Enzyme treated with PCMB (0·1 mM) tested immediately	27	—
	Enzyme treated with PCMB (0·1 mM) tested after 10 min. at 0°	34	—
	Enzyme treated with PCMB (0·1 mM) tested after 30 min. at 0°	20	—
	Enzyme treated with PCMB (0·1 mM) tested after 60 min. at 0°	0	—
3	Enzyme treated with PCMB (0·1 mM) for 5 min. at room temperature	0	—
	Enzyme mixed with substrate before addition of PCMB	38	—
	Enzyme mixed with substrate and 0·1 mM-FAD before addition of PCMB	40	—
4	Enzyme treated with PCMB (0·1 mM) tested at once	65	—
	Enzyme treated with PCMB then dialysed 17 hr. against 0·1 M-phosphate buffer (pH 7·4) at 1°	52	—
	Enzyme treated with PCMB, GSH (10 mM) added, left for 1 hr. at 15°, and dialysed against 0·1 M-phosphate buffer (pH 7·4), for 17 hr. at 1°	120	—
5	Enzyme treated with GSH (10 mM) for 1 hr. at room temperature dialysed against 0·01 M-phosphate buffer (pH 7·4), for 17 hr. at 1°	280	—
	Enzyme as above treated with PCMB (0·1 mM) for 45 min. at room temperature	130	—
	Enzyme after PCMB treatment re-treated with GSH (10 mM) and tested after dialysis for 17 hr. at 1°	200	—
6	Enzyme treated with PCMB (0·1 mM) tested at once	—	70
	Enzyme left 30 min. room temp. with PCMB (0·1 mM) dialysed for 17 hr. at 1° against 0·01 M-phosphate buffer	—	16
	Enzyme similarly treated as above except that GSH (10 mM) was added after 30 min. incubation with PCMB	—	72
	Enzyme similarly treated as above, except that cysteine (10 mM) was added after 30 min. incubation with PCMB	—	61

glutathione (GSH) was added within a short time after the addition of the PCMB; when added 45 min. after PCMB, considerable though not complete reactivation was obtained. In similar experiments it was found that cysteine could replace GSH. The enzyme can be partially protected against inhibition by PCMB by preincubation with the substrate (Table 2), suggesting that an –SH group is concerned with the attachment of substrate to enzyme.

In agreement with these results was the observation that GSH was capable of restoring activity which had been lost during various stages in the purification (Table 1), and what was more interesting was the observation that the enzyme activity, as determined with phenazine, appeared to be more adversely affected than that with cytochrome *c* as electron acceptor. This phenomenon has also been observed during the ageing of enzyme preparations kept at 0°. The activity of the enzyme, as assayed by phenazine, has often been found to decrease appreciably while the activity with cytochrome *c* has decreased to a much smaller extent. When this has happened, treatment with GSH restored the former activity fully, and had a much smaller effect on the latter. In a typical experiment the activity of an enzyme, as assayed against phenazine and cytochrome *c*, decreased by 55 and 20% respectively on ageing; after treatment with GSH the activity was fully restored with phenazine but was not altered with cytochrome *c*. In these experiments, excess of GSH was removed by dialysis to prevent its interfering in the assay.

These changes in the activity of the enzyme against the different electron acceptors, the greater instability of the enzyme with phenazine and the differential effect of GSH in restoring the activity suggest that an –SH group additional to that required for substrate-binding may be necessary for the interaction of phenazine with the enzyme.

Inhibitors. The enzymic reduction of either phenazine or cytochrome *c* in the presence of L-galactono-γ-lactone was unaffected by most metal-binding agents. Cyanide, ethylenediaminetetraacetic acid, pyrophosphate, azide and αα′-dipyridyl in mM concentrations were without effect on enzyme activity. No inhibition with these reagents was observed after a 15 min. preincubation treatment with the enzyme at room temperature. *o*-Phenanthroline (1:10-phenanthroline), however, inhibited the enzyme, and this inhibition was increased by prior incubation with the inhibitor (Table 3). None of the ions Mn^{2+}, Zn^{2+}, Fe^{3+} or MoO_4^{2-} had any effect on enzyme activity. Antimycin *A* in a concentration of 10 μg./ml. of reaction solution did not inhibit the enzyme.

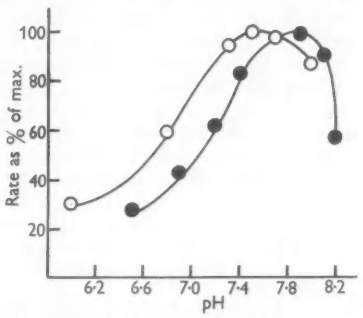

Fig. 2. Effect of pH on the activity of the enzyme with phenazine and cytochrome *c* as electron acceptors. ○, With phenazine. The reaction mixture was contained in a total vol. of 6 ml. 0·1 M-Phosphate buffer (4·5 ml.) at stated pH, 1 mg. of phenazine, and 12 mg. of L-galactono-γ-lactone; temp. 37°; ●, with cytochrome *c*. The reaction mixture was contained in a total vol. of 3 ml. 0·01 M-Phosphate buffer (2·0 ml.) at stated pH, 1·5 mg. of cytochrome *c* and 6 mg. of L-galactono-γ-lactone; temp. 17°.

Table 3. *Inhibition of L-galactono-γ-lactone dehydrogenase by o-phenanthroline*

Expt.	*o*-Phenanthroline added (mM)	Treatment	Inhibition (%) Phenazine reaction	Inhibition (%) Cytochrome reaction
1	0·5	Tested at once	—	16
		Preincubation for 30 min. at room temp.	—	26
	1	Tested at once	—	18
		Preincubation for 15 min. at room temp.	—	35
	2	Tested at once	—	31
		Preincubation for 30 min. at room temp.	—	50
		Preincubation for 60 min. at room temp.	—	55
2	1	Tested at once	35	—
		Preincubation for 10 min. at room temp.	35	—
	5	Preincubation for 10 min. at 37°	57	—
		Preincubation for 30 min. at 37°	100	—

pH *and activity*. With phosphate buffer the optimum pH for the reaction with the solubilized enzyme was about 7·8–7·9 at 17° with cytochrome *c* as electron acceptor, whereas with phenazine at 37° the optimum was 7·4–7·7 (Fig. 2). With mitochondrial preparations from peas the optimum pH found was about 7·5–7·6 at 37° (Mapson *et al.* 1954).

Substrate-affinity constants. The Michaelis constant for the substrate L-galactono-γ-lactone was determined by the method of Lineweaver & Burk (1934), with phenazine and cytochrome *c* as electron acceptors at 17°; with phenazine at pH 7·4 the constant was 4 mM and with cytochrome *c* at pH 7·8 was 2 mM. These constants are about 10 times that found for the reaction as catalysed by intact mitochondria prepared from pea seedlings (Mapson *et al.* 1954). The reason for this is unknown. One possibility is that during solubilization and extraction from the mitochondria minor changes are produced in the structure of the enzyme which are reflected in a reduced affinity of enzyme for substrate.

Specificity. The solubilized enzyme appears to be specific for L-galactono-γ-lactone. The enzyme as isolated from cauliflower florets failed to catalyse the reduction of cytochrome *c* in the presence of D-galactono-γ-lactone, D-glucono-γ-lactone, D-glucurono-γ-lactone, D-gulono-γ-lactone, L-gulono-γ-lactone, D-mannono-γ-lactone or L-galactonic acid, when tested at a concentration of 10 mM. In an equivalent concentration D-altrono-γ-lactone reduced cytochrome *c* at a rate less than one-tenth of that observed with L-galactono-γ-lactone.

Further studies with D-galactono-γ-lactone and D-gulono-γ-lactone have shown that these lactones inhibit competitively the oxidation of L-galactono-γ-lactone by the dehydrogenase with cytochrome *c* as electron acceptor. Both lactones inhibited the reaction to about the same extent; there was about a 20% reduction in rate when they were added in twice the molecular concentration (40 mM) to the substrate L-galactono-γ-lactone. Results with D-gulono-γ-lactone are shown in Fig. 3. These results are of interest in the light of the earlier work of Mapson *et al.* (1954) and are discussed below.

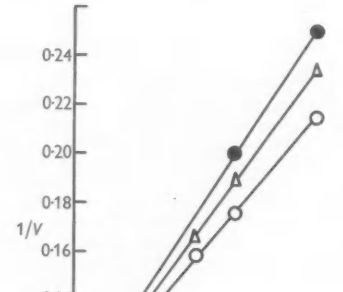

Fig. 3. Competitive inhibition of L-galactono-γ-dehydrogenase by D-gulono-γ-lactone. The reaction mixture was contained in a total vol. of 3 ml. 0·01 M-Phosphate buffer (pH 7·8) (2 ml.), cytochrome *c* 1·5 mg., L-galactono-γ-lactone and D-gulono-γ-lactone at the concentrations indicated; temp. 17°. D-Gulono-γ-lactone: ●, 7 mg./ml. of reaction mixture; △, 3·5 mg./ml.; ○, 1·75 mg./ml. *V* = units of enzyme activity. *S* = concn. of L-galactono-γ-lactone (mg./ml. of reaction solution).

Isherwood *et al.* (1954) and Mapson *et al.* (1954) both observed the inhibition of formation of ascorbic acid by DL-glyceraldehyde, the former workers with growing cress seedlings and the latter when studying the conversion of L-galactono-γ-lactone into ascorbic acid by pea mitochondria. These results have now been supplemented by the observation that DL-glyceraldehyde in a concentration of 10 mM and with the substrate in a concentration of 15 mM reduced the enzyme activity by about 30%, an effect of similar magnitude to that obtained by Mapson *et al.* (1954).

Formation of ascorbic acid by cauliflower mitochondria

To determine whether the high specificity of the solubilized enzyme was also a characteristic of the enzyme in the mitochondria, experiments were carried out with mitochondria prepared from the florets by the method previously used for preparing pea mitochondria (Mapson *et al.* 1954). The mitochondrial preparations so obtained contained a very active ascorbic acid-oxidizing system, so that the formation of ascorbic acid from L-galactono-γ-lactone could not be detected unless an enzyme preparation (Mapson, 1953) from dried peas containing glutathione reductase, dehydroascorbic acid reductase and malic and *iso*citric enzymes, together with triphosphopyridine nucleotide (TPN), glutathione and malate or *iso*citrate, was added to maintain the ascorbic acid in the reduced form. Under such conditions the formation of ascorbic acid was readily observed (Fig. 4). It was more convenient to use the alternative procedure of following the reaction by determining the rate of formation of 2:3-dioxo-L-gulonic acid, for testing the activity of the different lactones. The results

Table 4. *Formation of 2:3-dioxo-L-gulonic acid from certain aldonic acid lactones by cauliflower or pea mitochondria*

Source	Lactone added (mg./ml. of reaction mixture)	2:3-Dioxo-L-gulonic acid formed (μg./ml. of reaction mixture/hr.)	Relative rate (L-galactono-γ-lactone = 100)
Cauliflower florets	L-Galactono-γ-lactone	184	100
	L-Gulono-γ-lactone	0	0
	D-Altrono-γ-lactone	28	15
	D-Arabono-γ-lactone	14	7
Cauliflower florets	L-Galactono-γ-lactone	88	100
	D-Mannono-γ-lactone	0	0
	D-Gulono-γ-lactone	0	0
	D-Galactono-γ-lactone	0	0
Fresh green peas	L-Galactono-γ-lactone	320	100
	D-Altrono-γ-lactone	192	60
	D-Arabono-γ-lactone	200	63
Cotyledons from 5-day-germinated pea seeds	L-Galactono-γ-lactone	135	100
	D-Altrono-γ-lactone	95	70
	D-Arabono-γ-lactone	90	67

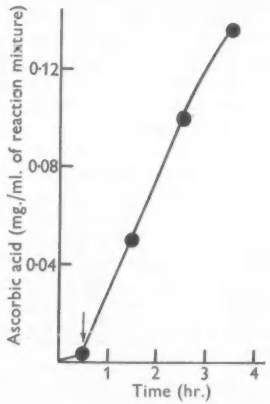

Fig. 4. Formation of ascorbic acid by cauliflower mitochondria. Mitochondria were suspended in 0·4 M-sucrose, 0·1 M-phosphate (pH 7·4), containing glutathione reductase and dehydroascorbic acid-reductase enzyme systems from peas (see text). In addition, TPN (60 μg./ml.), DL-malate (1 mg./ml.) and GSH (1 mg./ml.) were added at zero time; temp. 37°. ↓ denotes addition of L-galactono-γ-lactone (1 mg./ml.).

(Table 4) showed that the mitochondrial preparations behaved similarly to the solubilized enzyme; there was the same high specificity towards the lactones, L-galactono-γ-lactone being the only one which was oxidized at a high rate. D-Altrono-γ-lactone and D-arabono-γ-lactone were oxidized only slowly and the other lactones tested, including L-gulono-γ-lactone, were not oxidized. There was no oxidation of L-galactonic acid.

When these experiments were repeated with mitochondria prepared from fresh green peas, or from the cotyledons of 5-day-germinated pea seeds, both D-altrono- and D-arabono-γ-lactones were oxidized at about 60% of the rate of L-galactono-γ-lactone. A similar result to this was obtained in experiments with the solubilized enzyme from green peas, where, with cytochrome *c* as acceptor, both lactones were oxidized at about half the rate of L-galactono-γ-lactone. It seems probable that the enzyme as it occurs in cauliflower and peas differs in degree in its specificity.

Prosthetic group

There is no evidence that either diphosphopyridine nucleotide (DPN) or TPN participates in the reaction. Neither DPN nor TPN increased the rate at which either phenazine or cytochrome *c* was reduced, nor were the coenzymes reduced (as determined by the increase in absorption at 340 mμ) in the presence of enzyme and substrate.

Evidence was obtained which suggested that a flavin group is involved in the reaction with cytochrome *c*. The enzymic reduction of cytochrome *c* was strongly inhibited by either Atebrin or riboflavin, known flavoprotein inhibitors (Haas, 1944; Wright & Sabine, 1944; Hellerman, Lindsay & Bovarnick, 1946) at concentrations of 0·1–1·0 mM (Table 5). Of the two inhibitors, riboflavin was the most active, a finding in agreement with the results of Singer & Kearney (1950) on the flavo-enzyme L-amino acid oxidase. Equally in agreement with their results was our failure to diminish the inhibitory effect of Atebrin (3-chloro-7-methoxy-9-(1-methyl-4-diethylaminobutylamino)acridine dihydrochloride or riboflavin by the addition of flavinadenine dinucleotide (FAD) or flavin mononucleotide (FMN).

In contrast with these results was the observation that the reaction with phenazine was not

inhibited by either Atebrin or riboflavin, although acriflavine, especially if incubated with the enzyme before test, caused some inhibition (Table 5). This inhibition was reduced with FAD, although FAD did not stimulate the activity of the unincubated enzyme. The decrease of inhibition with FAD appeared to be due, however, to a protective effect on the enzyme during incubation at 37° rather than to any reversal of the inhibition.

Attempts to resolve the enzyme reversibly into apoenzyme and prosthetic group have so far met with little success. The enzyme was very sensitive to changes in pH; when the pH of the solution was decreased to 5·0 or below, in the presence or absence of ammonium sulphate, almost complete inactivation occurred within a period of 5 min. at 0°. This inactivation could not be reversed by the addition of FAD, FMN or a boiled extract of the enzyme. Attempts were also made to remove a prosthetic group by less drastic acid treatment, e.g. by dialysis against sodium acetate–acetic acid buffers (0·01 M) at a pH of 4·5–5·0 for periods of 2–17 hr. at 0°, or by precipitating the enzyme within this pH range with ammonium sulphate. In all these attempts no appreciable reactivation was obtained with either FAD or FMN.

Absorption spectrum. The absorption spectrum of the most highly purified preparations so far obtained (specific activity 300–340 cytochrome units) showed evidence of a flavin component with maxima at 375 and 455 mμ (Fig. 5). These absorption bands disappeared on the addition of L-

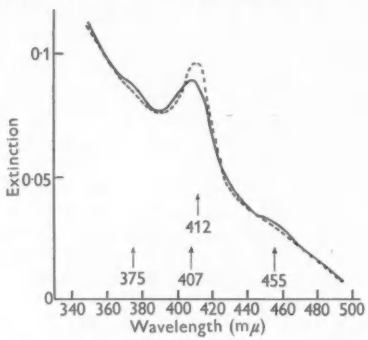

Fig. 5. Absorption spectra of an enzyme preparation (specific activity 298) before and after the addition of L-galactono-γ-lactone (1 mg./ml.). —, Oxidized; - - - -, reduced.

Table 5. *Effect of Atebrin, riboflavin and acriflavine on enzyme activity*

Expt.	Inhibitor	Concn. (mM)	Treatment	Activity (% of control) Phenazine reduction	Cytochrome *c* reduction
1	Atebrin	0·1	Tested after addition	100	85
		1	Tested after addition	93	41
		1	Tested after addition	100	35
		2·5	Tested after addition	—	11
		2·5 + FAD 0·01	Tested after addition	—	11
		2·5 + FAD 0·1	Tested after addition	—	13
		6	Incubated with enzyme for 10 min. at 37° before test	96	—
		6	Incubated with enzyme for 60 min. at 37° before test	93	—
2	Atebrin	1	Tested after addition	—	56
		1 + FMN 1	Tested after addition	—	55
3	Riboflavin	1	Tested after addition	100	—
		2	Incubated with enzyme for 10 min. at 37°	97	—
		0·1	Tested after addition	—	56
		0·2	Tested after addition	—	44, 38
		0·2 + FAD 0·1	Tested after addition	—	48
		0·5	Tested after addition	—	34
4	Riboflavin	0·1	Tested after addition	—	66
		0·1 + FMN 1	Tested after addition	—	76
		0·1 + FMN 10	Tested after addition	—	60
5	Acriflavine	0·1	Incubated with enzyme for 10 min. at 37° before test	63	—
		1	Incubated with enzyme for 20 min. at 37° before test	50	—
		3	Incubated with enzyme for 15 min. at 37° before test	39	—
		3 + FAD 1	Incubated with enzyme for 15 min. at 37° before test	79	—
		1	Tested immediately	—	66

galactono-γ-lactone, and the difference spectrum obtained was typical of many flavo-protein enzymes. The oxidized spectrum showed an additional absorption band in the Soret region with a peak at 407 mμ, which on reduction with the lactone increased and the maximum shifted to 412 mμ. When dithionite was added this absorption band was still further intensified, and in addition two bands at 518–520 mμ and at 550–555 mμ were observed. This suggests that a haem compound may be present as an additional component (cf. Mahler, Mackler, Green & Bock, 1954).

Identification of flavin. The most active enzyme extracts so far obtained showed a weak, yellowish fluorescence in ultraviolet light, which was increased three- to four-fold after acidification with trichloroacetic acid and neutralization. Estimation of riboflavin by the lactobacillus test (Clegg, Kodicek & Mistry, 1952) was positive and showed, in the latter stages of purification, that the proportion of flavin to protein rose as the specific activity increased. With the purest preparations so far obtained (specific activity 300) a mole of flavin was associated with about 800 kg. of protein, which would indicate either that the content of flavin in the enzyme is much lower than is usually found in flavoproteins, or, what is more likely, that much inert protein is still present. We hope to continue the purification when larger amounts of enzyme become available.

An analysis of the flavin component of the enzyme has been carried out by paper chromatography. After the enzyme had been dialysed against distilled water for 3 hr. at 0°, the flavin was split from the holoenzyme either with acid at pH 2·5 or by heat-denaturation. The solution, centrifuged to remove protein, was concentrated *in vacuo* to dryness, and the residue extracted with 50% ethanol to remove the flavin. This procedure was found to be essential in order to free the small amount of flavin present from inorganic salts. The ethanol extract was then chromatographed with two solvents. With either a solvent mixture of butanol–acetic acid–water (4:1:5, by vol.), or a mixture of 5% (w/v) disodium hydrogen phosphate and 3-methylbutan-1-ol (Carter, 1950) and flavin-adenine dinucleotide (FAD), flavin mononucleotide (FMN) and riboflavin as markers, the flavin component of the enzyme was resolved into two spots, one with the same R_F as FMN (R_F 0·1 in the first solvent and 0·5 in the second) and the other with an R_F corresponding to riboflavin (R_F 0·3 in the first solvent and 0·3 in the second). No FAD could be detected. These results were confirmed by using a mixed chromatographic analysis, whereby the addition of FMN or riboflavin to the flavins obtained from the enzyme gave a chromatogram identical with that obtained from the flavins of the enzyme alone.

The presence of FMN after splitting was confirmed by a positive reaction with the FMN-dependent apoenzyme of TPNH cytochrome *c* reductase prepared from yeast by the method of Haas, Horecker & Hogness (1940) and the absence of FAD was likewise confirmed by the inability of the split flavins to reactivate the apoenzyme of D-amino acid oxidase (Huennekens & Felton, 1957).

Fluorimetric assay of the two flavins by the method described by Burch (1957) showed that FMN was present in a concentration three times that of riboflavin. At present it is not clear whether the riboflavin found is an impurity associated with the enzyme (though this seems unlikely in view of the steps used in its purification), or whether it originated from a breakdown of the flavin prosthetic group during the splitting from the holoenzyme, similar to that described by Mahler, Sarkar, Vernon & Alberty (1952) for DPN cytochrome *c* reductase.

Phosphate and activity

The phenazine-reducing system did not exhibit maximal activity except in the presence of phosphate. It is not certain whether the requirement for phosphate is absolute, as even preparations dialysed to reduce phosphate may contain enough to account for the residual activity. The results (Table 6) indicate that maximal activity was attained at a level of approximately 0·1 M-phosphate. When either veronal or 2-amino-2-hydroxymethylpropane-1:3-diol (tris) was used as buffer, the activity depended on the presence of phosphate, but the maximal activity obtained with the added phosphate was less than that of the phosphate alone. This may be due to an inhibitory effect of these buffers in high concentrations, although they were not inhibitory at mM levels.

Pyrophosphate and arsenate, among other salts tested, were even more effective than phosphate, especially when added in the presence of tris buffer; sodium chloride was without effect.

The stimulative effect of phosphate on the flavo-enzymes, xanthine and aldehyde oxidases, in their reactions with certain electron acceptors, has been described by Mackler, Mahler & Green (1954) and by Mahler *et al.* (1954), and a similar effect to that reported here (of phosphate on the enzymic reduction of phenazine by succinic dehydrogenase) has been observed by Singer, Kearney & Zastrow (1955).

Inhibition of reduction of cytochrome c *by anions.* In contrast with the results with phenazine the reduction of cytochrome *c* was inhibited by raising the concentration of phosphate. This inhibition was, however, not specific for phosphate, for

Table 6. *Stimulative effect of phosphate and other salts on the reduction of phenazine*

Arsenate, chloride, phosphate and pyrophosphate were added as sodium salts neutralized to pH 7·4 where necessary before addition.

Expt.	Buffer	Concn. (M)	Salt	Concn. added (M)	Enzyme activity (phenazine units)
1.	Phosphate	0·01	—	Nil	1·20
		0·03	—	Nil	1·95
		0·04	—	Nil	2·35
		0·10	—	Nil	3·25
		0·13	—	Nil	3·55
		0·16	—	Nil	3·15
2. Enzyme (3 ml.) dialysed overnight against 2 l. of 0·1 M-tris buffer (pH 7·4)	Tris	0·08 (pH 7·4)	—	Nil	0·65
		0·08	Phosphate	0·046	1·05
		0·08		0·06	1·25
		0·08		0·08	2·05
		0·08		0·125	2·00
		0·08		0·133	2·05
		—		0·09	2·85
		—		0·15	2·85
3.	Veronal	0·05 (pH 7·4)		0·008	0·60
		0·05	Phosphate	0·10	1·20
		—		0·10	1·95
4.	Tris	0·05 (pH 7·4)	—	Nil	0·50
		0·05	Phosphate	0·1	1·30
		0·05	Pyrophosphate	0·1	2·60
		0·05	Arsenate	0·1	2·00
		0·05	Chloride	0·1	0·20
		—	Phosphate	0·1	1·80
			Pyrophosphate	0·1	2·20

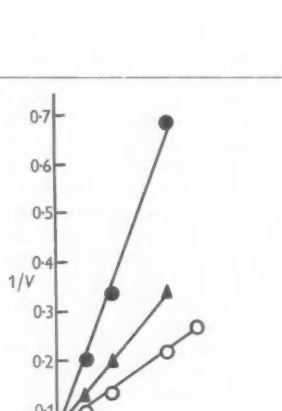

Fig. 6. Competitive nature of the inhibition by phosphate on the enzymic reduction of cytochrome *c*. Each solution contained enzyme, L-galactono-γ-lactone (2 mg./ml.) with varying amounts of cytochrome *c* and phosphate (pH 7·8); temp. 17°. Phosphate: ●, 0·025 M; ▲, 0·015 M; ○, 0·01 M. V = units of enzyme activity. S = concn. of cytochrome *c* (mg./ml. of reaction solution).

inhibition in varying degrees was observed with many anions. The inhibitory effect of several sodium salts was tested at a level of 0·02 M on the reaction with cytochrome *c* in the presence of 0·01 M-phosphate buffer (pH 7·8). The results showed that the inhibition (52 %) due to SO_4^{2-} ions was greatest, followed in descending order by NO_3^- (43 %), F^- (30 %), Cl^- (24 %), Br^- (21 %) and AcO^- ions (8 %). Sucrose, an un-ionized solute, had no inhibitory effect in concentrations as high as 0·4 M.

The inhibitory effect of anions on the activity of certain flavin enzymes has been noted by Theorell & Nygaard (1954) for the 'old yellow enzyme' and by Walaas & Walaas (1956) for D-amino acid oxidase, and pronounced effects of anions on the activity of DPN- and TPN-linked enzymes such as alcohol dehydrogenase and glucose 6-phosphate dehydrogenase have also been described (Theorell, Nygaard & Bonnichsen, 1954; Rutter & Rolander, 1955). The former workers found that the anions inhibit by increasing the dissociation of coenzyme from apoenzyme, thereby reducing velocity of the enzyme reaction. Lineweaver–Burk plots of $1/V$ against $1/S$, where S was equal to the concentration of cytochrome *c* at three different levels of phosphate, indicated that inhibition by phosphate was competitive (Fig. 6), and similar results were obtained with sodium chloride. This reversal of the inhibition by cytochrome *c* suggests that the anions affect the binding of this compound to the enzyme. It is of course possible that the anions may also affect the dissociation of coenzyme from apoenzyme.

DISCUSSION

The solubilization and isolation of L-galactono-γ-lactone dehydrogenase from a plant mitochondrial preparation adds further to our knowledge of the mechanism whereby L-ascorbic acid is synthesized *in vivo*. Of special interest in this respect was our observation of the high specificity of the enzyme. The inability of the enzyme to oxidize the closely related L-gulono-γ-lactone would indicate an enzymic specificity for the configuration of the groups on C (2) and C (3) of the aldonic acid lactone; in L-galactono-γ-lactone these are L and D respectively. In agreement with these results were the observations that the enzyme failed to catalyse the oxidation of D-mannono-, D-glucono-, D-galactono- or D-gulono-γ-lactone, in which the configuration on C (2) and C (3) is also different from that of L-galactono-γ-lactone, and in addition the configuration on C (5) is D, not L. D-Altrono-γ-lactone, which has the same configuration of the groups on C (2) and C (3) as L-galactono-γ-lactone but the opposite configuration on C (5), was oxidized only very slowly; this would indicate that the configuration of the groups on C (5) influences the rate of oxidation of the lactone.

The fact that essentially similar results to these were obtained with cauliflower mitochondria makes it improbable that during extraction and purification any changes had occurred in the enzyme protein which had altered its specificity. These results may be compared with those obtained in the earlier work with cress seedlings (Isherwood *et al.* 1954) and with pea mitochondria (Mapson *et al.* 1954). With growing cress seedlings L-galactono- and D-altrono-γ-lactone were converted into an ascorbic acid to about the same extent, the only difference being that L-ascorbic acid was formed with the first lactone and D-araboascorbic acid with the second. Under the same conditions L-gulono-γ-lactone as a percursor of L-ascorbic acid was only about one-tenth as effective as L-galactono-γ-lactone. With pea mitochondria D-altrono-γ-lactone was converted into an ascorbic acid at about one-half, and L-gulono-γ-lactone at only one-twenty-fifth, of the rate of conversion of L-galactono-γ-lactone.

The additional results given in this paper indicating a higher specificity of the enzyme from cauliflower as compared with peas suggests that the differences are due to real differences in the specificity of the enzymes in the different plants. It is unlikely that the results can be explained by the removal of waldenase-like enzymes during extraction and purification of the solubilized enzymes, which catalyse the interconversion of the lactones. Whatever may be the true explanation, it seems clear that with all plant enzymes so far examined L-galactono-γ-lactone is pre-eminent as a precursor of L-ascorbic acid.

Hassan & Lehninger (1956) reported that mitochondrial preparations from rat liver were able to form ascorbic acid from the free acid as easily as from the lactone. Neither in this work nor in the earlier work with pea mitochondria (Mapson *et al.* 1954) have we observed the formation of ascorbic acid from L-galactonic acid.

The nature of the electron path from the lactone to phenazine and cytochrome *c* cannot be determined until further purification of the enzyme is accomplished. Preliminary evidence, however, indicates that the paths to these two electron-acceptors are not identical. Inhibition of the cytochrome *c* reaction by Atebrin, acriflavine and riboflavin suggests that flavin is involved. The changes in the spectrum accompanying the reduction of the enzyme by substrate further support the theory that the enzyme is a flavoprotein. The reduction of phenazine, on the other hand, is insensitive to Atebrin or riboflavin, and it is thus conceivable that the phenazine reaction proceeds without flavin's being involved.

The requirement of L-galactono-γ-lactone dehydrogenase for inorganic phosphate for the reduction of phenazine is similar to the requirement of succinic dehydrogenase for phosphate (Singer *et al.* 1955). Another similarity between the two enzymes is the insensitivity of both to most metal-binding agents with the exception of *o*-phenanthroline (Singer, Kearney & Massey, 1956) and it may be that L-galactono-γ-lactone dehydrogenase is also a ferroflavoprotein. Singer *et al.* (1956) assumed that, with succinic dehydrogenase, electrons were transferred from succinate to flavin to iron and then to phenazine. Such a scheme is clearly inapplicable to the reduction of phenazine by L-galactono-γ-lactone dehydrogenase in view of the insensitivity of the reaction to Atebrin and riboflavin. In both *Bacterium tularense* (Wadkins & Mills, 1956) and in *Tetrahymena* (Eichel, 1956) it has been found that the reduction of phenazine by succinic dehydrogenase is Atebrin-insensitive, whereas the succinoxidase reaction is readily inhibited. Assuming the presence of flavin in the microbial dehydrogenases as in beef-heart dehydrogenase, it seems that in these, as well as in L-galactono-γ-lactone dehydrogenase, flavin may not be the initial electron acceptor but that electrons are transferred from substrate to a primary acceptor (conceivably metal) and then either to flavin or to phenazine. A scheme cannot be excluded, however, in which phenazine is positioned in an electron chain between a primary Atebrin-insensitive flavin component and a flavin which is Atebrin-sensitive. In any event, it appears likely that L-galactono-γ-lactone dehydrogenase

will fall into that class of flavin enzymes in which the active sites involve electron transport between different prosthetic groups on the same enzyme surface.

SUMMARY

1. An enzyme oxidizing L-galactono-γ-lactone to L-ascorbic acid has been extracted from cauliflower mitochondria and purified 12-fold from an acetone-dried powder.

2. Only phenazine methosulphate and cytochrome *c* of the various electron acceptors tested were reduced at a significant rate by the enzyme in the presence of substrate.

3. Phosphate stimulated the enzymic reduction of phenazine, but phosphate and other anions inhibited the corresponding reduction of cytochrome *c*.

4. The enzyme requires thiol groups for its activity; it was inhibited strongly by certain thiol inactivators and this inhibition was reversed by glutathione.

5. The enzyme was not inhibited by cyanide, ethylenediaminetetra-acetic acid, pyrophosphate, azide or $\alpha\alpha$-dipyridyl, but was inhibited by *o*-phenanthroline.

6. Evidence of the flavin nature of the prosthetic group of the enzyme was obtained from the absorption spectrum, the presence of flavin in the most active preparations and the inhibition of the enzyme by riboflavin and Atebrin.

7. The substrate specificity of the enzyme was high; of the several aldonic acid lactones tested, L-galactono-γ-lactone was the only one oxidized at a high rate, D-altrono-γ-lactone was oxidized only very slowly and none of the others were oxidized.

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Biosynthesis of Ergothioneine by *Claviceps purpurea*

3. THE INCORPORATION OF LABELLED HISTIDINE*

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Degradation of ergothioneine and histidine isolated from cultures of *Claviceps purpurea* grown in the presence of [2-^{14}C]acetate has revealed a similar distribution of labelling in the six-carbon structure common to both these compounds (Heath & Wildy, 1956*a*, *b*). These results indicated that this fungus forms ergothioneine by methylation and sulphuration of histidine.

Subsequent work (Wildy & Heath, 1956, 1957) revealed that [2(ring)-^{14}C]histamine would not serve as a precursor of the imidazole nucleus of either histidine or ergothioneine. Various metabolites of histamine, imidazoles possessing modified side chains, also failed to provide the requirements of an ergothioneine precursor.

In order to confirm that histidine was a direct precursor of ergothioneine, experiments were carried out with histidine labelled both in the side chain and the imidazole ring. A preliminary report of this work has already been published (Heath & Wildy, 1957).

EXPERIMENTAL

Organism. The culture of *Claviceps purpurea* (no. 44613) was originally obtained from the Commonwealth Mycological Institute, Kew, London, and was maintained as described previously (Heath & Wildy, 1956*b*).

DL-[α-^{14}C]*Histidine.* This was obtained from Tracer Lab, Boston (activity 0·5 mc/m-mole).

L-[2(*ring*)-^{14}C]*Histidine.* This was obtained from the Radiochemical Centre, Amersham (activity 4 mc/m-mole).

The following materials and methods used have been described previously (Heath & Wildy, 1956*b*): activated alumina; Zeo-Karb 225; Oxoid Membrane Filters; determination of radioactivity; sterilization; culture of organism; detection and estimation of ergothioneine.

Radioautography of chromatograms. Two-dimensional chromatograms were prepared on 10 in. squares of Whatman no. 4 paper by the frame technique of Datta, Dent & Harris (1950). A glass and polythene tank assembly was used; the tank and polythene-coated trough were purchased from Aimer Products Ltd., London, N.W. 1, and the all-polythene frame was made by Mr P. R. E. Wallace of this Department. The radioactive solution (10–30 μl.) was applied to the paper, the dried spot was counted with a mica end-window Geiger tube and the time required for exposure of the subsequently developed chromatogram to the X-ray film was calculated on the basis of this activity. Under our experimental conditions of counting, an exposure equivalent to at least $1{\cdot}5 \times 10^6$ total counts was found to give a satisfactory radioautograph (e.g. a spot giving 1050 counts/min. required a 24 hr. exposure).

The chromatograms were developed with phenol (80% aq. soln.) in the presence of aq. 0·1 N-NH_3 soln., followed by *n*-butanol–acetic acid–water (60:15:25) and then dried in air until no odour of solvent remained. An identification number was written on the edge, and the corners of the square were marked with a radioactive ink so that the paper chromatogram could be subsequently superimposed on the radioautograph. Each chromatogram was then placed in contact with a 10 in. × 12 in. sheet of Kodirex Increased Speed X-ray Film (Kodak Ltd., Kingsway, London, W.C. 2) and left for the required time. The film was then developed for 5 min. at 60° F. in a solution consisting of: *p*-methylaminophenol sulphate (Metol), 2·2 g.; quinol, 8·8 g.; $Na_2SO_3,7H_2O$, 144 g.; $Na_2CO_3,10H_2O$, 130 g.; KBr, 4 g.; water to 1 l. The film was then washed and fixed in a solution of 25% $Na_2S_2O_3,5H_2O$. The paper chromatograms were then treated with ninhydrin.

Growth of C. purpurea *on a medium containing* [α-^{14}C]-*histidine.* DL-[α-^{14}C]Histidine (0·1 mc, 30·4 mg.) was added to 600 ml. of the mannitol–ammonium succinate medium which was then distributed between five 350 ml. Erlenmeyer flasks. Four control cultures were also grown, two containing the basic medium and two supplemented with 6·2 mg. of histidine. The pH of the medium before growth was 5·8. After sterilization the cultures were grown on a rotary shaker for 12 days and were then harvested by filtration through Oxoid Membrane Filters and washed with water; the five cultures containing labelled histidine were harvested together and the four controls separately. The pH of the culture filtrate was 7·6.

Estimation of activity in the culture medium before and after growth. A portion (0·1 ml.) was taken from the culture medium before growth; it was evaporated to dryness in a small Quickfit flask and totally oxidized, the CO_2 being collected as $BaCO_3$ which was plated and counted at infinite thickness in a polythene planchet. A portion (0·2 ml.) of the culture filtrate was evaporated to dryness and oxidized in the same way. The culture filtrate was passed through a column of Zeo-Karb 225, H^+ form; 0·1 ml. of the culture filtrate and 0·1 ml. of the eluate from the column were estimated for activity by counting as infinitely thin film.

Ergothioneine was either estimated or detected chromatographically in the control cultures in order to ensure that the fungus was still producing ergothioneine in the presence of added histidine.

* Part 2: Wildy & Heath (1957).

Isolation of erogthioneine from the [α-^{14}C]*histidine culture.* L-Ergothioneine hydrochloride (90 mg.) was added to the total growth, which was then extracted three times with hot water (total vol. 120 ml.). The residue was then extracted twice with boiling water (total vol. 140 ml.) and filtered through an Oxoid Membrane. The combined extracts were deproteinized and chromatographed on Zeo-Karb 225 and alumina as described previously (Heath & Wildy, 1956*b*) for the isolation of [^{14}C]ergothioneine from the growth of *C. purpurea* on [2-^{14}C]acetate. The isolated ergothioneine was crystallized to constant activity. A final yield of 39·1 mg. of highly radioactive ergothioneine was obtained. This was diluted by adding to it ergothioneine hydrochloride (200 mg.). The material was dissolved in water and chromatographed on a column of Zeo-Karb 225, H^+ form, to convert the ergothioneine hydrochloride into the free base. The final yield of ergothioneine (free base) was 166·1 mg. (activity 1580 counts/min./mg. of C). This ergothioneine was used for the chemical degradation.

Degradation of [^{14}C]*ergothioneine isolated from the* [α-^{14}C]*histidine culture: oxidation of formic acid.* The ergothioneine (68·8 mg.) was degraded by the method described by Heath & Wildy (1956*b*), with the exception of the oxidation of formic acid obtained from the fission of the imidazole ring, which was carried out as follows. The aqueous solution remaining after ether extraction of the ethyl 4:5-dibenzamidopent-5-enoate, which contained sodium formate derived from the imidazole C-2 of ergothioneine, was heated to remove the dissolved ether. The remaining solution was transferred to a Quickfit flask, connected through a double-surface reflux condenser to a $Ba(OH)_2$ trap; 2 N-H_2SO_4 (6 ml.) was added, the solution was boiled and a stream of N_2 was passed slowly through the apparatus until no more CO_2 was evolved. The formic acid was distilled from this residual solution by boiling in a CO_2-free apparatus; the formic acid and water distilled over together and in this way a separation from traces of residual ethyl dibenzamidopentenoate was achieved. The formic acid was oxidized in a CO_2-free apparatus by boiling for 1 hr. with 20 ml. of acid mercuric sulphate reagent (mercuric sulphate, 10 g.; CO_2-free water, 80 ml.; conc. H_2SO_4, 20 ml.). The CO_2 from the oxidation of formic acid was displaced by N_2 and collected as $BaCO_3$. This was filtered off and counted as a thin layer on the filter paper; yield of $BaCO_3$, 4 mg.

Investigation of the distribution of labelling in the amino acids of the protein of C. purpurea *grown in the presence of* [α-^{14}C]*histidine.* A small portion of the ethanol-insoluble 'protein' from *C. purpurea* was hydrolysed by heating in a sealed capillary in 6 N-HCl at 100° for 16 hr. and then evaporated to dryness. A portion of the hydrolysate was chromatographed on a 10 in. square of Whatman no. 4 paper in phenol–aq. NH_3 soln. and butanol–acetic acid. A radioautograph was made of the resulting chromatogram.

Growth of C. purpurea *on a medium containing* [2(*ring*)-^{14}C]*histidine.* L-[2(ring)-^{14}C]Histidine (0·1 mc, 3·9 mg.) was added to 600 ml. of the mannitol–ammonium succinate medium, which was distributed between five 350 ml. Erlenmeyer flasks (120 ml. of medium/flask) and grown together with controls as described in the experiment with [α-^{14}C]histidine.

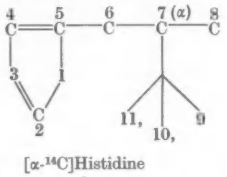
Ergothioneine was detected and estimated in the controls and ergothioneine hydrochloride (90 mg.) was added as carrier to the main growth; the labelled ergothioneine was then isolated by ion-exchange and alumina chromatography. The ergothioneine was crystallized to constant activity, yielding finally 33·3 mg. (activity 6840 counts/min./mg. of C). This ergothioneine was not degraded. L-Ergothioneine hydrochloride (204·3 mg.) was added to the mother liquors from the ergothioneine recrystallizations and the resulting solution was chromatographed on a column of Zeo-Karb 225 to convert the ergothioneine hydrochloride into the free base. The ergothioneine was crystallized to constant activity from aq. ethanol, yielding finally 97·5 mg. (activity 767 counts/min./mg. of C). This ergothioneine was used for degradation.

Degradation of [^{14}C]*ergothioneine isolated from the* [2(*ring*)-^{14}C]*histidine culture.* Ergothioneine (68·8 mg.) was degraded by the method described for the degradation of ergothioneine derived from [α-^{14}C]histidine. A portion of the ethanol-insoluble 'protein' from this culture was hydrolysed, the hydrolysate chromatographed and a radioautograph made of the chromatogram.

RESULTS

The distribution of radioactivity in the ergothioneine isolated from the [α-^{14}C]histidine culture and that isolated from the [2(ring)-^{14}C]histidine culture is shown in Table 1.

Table 1. *Distribution of radioactivity in ergothioneine isolated from cultures of* Claviceps purpurea *grown on media containing* [α-^{14}C]*histidine or* [2(*ring*)-^{14}C]*histidine*



Carbon atoms	[α-^{14}C]Histidine: Counts/min./mg. of C	[α-^{14}C]Histidine: Total counts/min.	[α-^{14}C]Histidine: Percentage of total	[2(ring)-^{14}C]Histidine: Counts/min./mg. of C	[2(ring)-^{14}C]Histidine: Total counts/min.	[2(ring)-^{14}C]Histidine: Percentage of total
2, 4, 5, 6, 7, 8, 9, 10, 11	1580	14 220	100·0	767	6900	100·0
9, 10, 11	0	0	0	27	81	1·2
4, 5, 6, 7, 8	2860	14 300	100·6	0	0	0
2	0	0	0	6554	6554	95·0

It can be seen that all the activity in the ergothioneine isolated from the [α-^{14}C]histidine culture was concentrated in the five-carbon chain (4, 5, 6, 7, 8). The carbon atoms of the betaine (9, 10, 11) and the imidazole C-2 were completely inactive. A radioautograph of a chromatogram of a protein hydrolysate from this [α-^{14}C]histidine culture showed that histidine had been partially degraded to glutamic acid. No activity was detected in the other amino acids.

Of the activity originally present in the [α-^{14}C]-histidine culture medium, 19% remained in the culture filtrate after growth, and of this 98·5% was adsorbed when the culture filtrate was applied to a Zeo-Karb column.

From Table 1 it can be seen that 95% of the activity in the ergothioneine isolated from the [2(ring)-^{14}C]histidine experiment was present in the imidazole ring C-2. A small activity was detected in the carbon atoms of the methyl groups of the betaine (9, 10, 11), whereas the five carbon atoms of the chain (4, 5, 6, 7, 8) were completely unlabelled.

The radioautograph of the chromatogram of hydrolysed protein revealed a very intense spot corresponding to histidine and three other weaker spots corresponding to methionine, serine and glycine.

Of the activity originally present in this [2-(ring)-^{14}C]histidine culture, 43% remained in the culture medium after growth. When this culture filtrate was applied to a column of Zeo-Karb 225, 39% of the activity was retained by the column.

DISCUSSION

The results of these experiments fully confirm that ergothioneine is formed by the fungus *Claviceps purpurea* directly from histidine without fission of the imidazole ring or alteration to the aliphatic side chain. The incorporation of [α-^{14}C]histidine into ergothioneine by *C. purpurea* clearly showed that histidine was a precursor, but it did not establish that the intact imidazole ring of histidine was directly utilized for the formation of ergothioneine. The possibility existed that ring fission of histidine could have occurred, followed by the reincorporation of a one-carbon compound linked through sulphur such as the $-SCH_3$ group of methionine. However, the ergothioneine isolated from the culture grown in the presence of ring-labelled histidine was highly labelled and the activity was concentrated in the imidazole C-2. The slight activity in the carbon atoms of the betaine showed that there had been some metabolism of the labelled histidine by the fungus, resulting in the liberation of labelled one-carbon fragments. This was confirmed by the radioautograph of the chromatogram of hydrolysed protein which showed the presence of radioactive methionine, serine and glycine. The high degree of incorporation of labelling into the imidazole C-2 of ergothioneine, however, showed that the intact imidazole ring of histidine had been incorporated into ergothioneine; had this labelling arisen by reincorporation from the one-carbon pool, the activity in this carbon atom would have been only of the order of that detected in the carbon atoms of the betaine.

Melville, Eich & Ludwig (1957) have recently studied the biosynthesis of ergothioneine by *Neurospora crassa* and they have shown on the basis of the high degree of incorporation of labelled histidine into the isolated ergothioneine that this organism also derived its ergothioneine by synthesis from histidine. As our results and those of Melville *et al.* (1957) are in complete agreement for two different fungi it would appear that the formation of ergothioneine from histidine is the normal pathway of this synthesis.

Some information on the metabolism of histidine by this fungus is obtained from the distribution of isotope in the culture broth after growth. All the amino acids, including histidine and glutamic acid, are retained by the ion-exchange resin, whereas the carboxylic acids, including formic acid, are not adsorbed. In the growth containing [2(ring)-^{14}C]-histidine, the liberation of one-carbon fragments not attached to nitrogen was demonstrated by the fact that 61% of the isotope was in the non-basic fraction, whereas in the experiment with [α-^{14}C]-histidine only 1·6% of the isotope was in this similar fraction. Radioautographs of two-dimensional chromatograms of the hydrolysed proteins from these experiments showed the presence of labelled methionine, serine and glycine derived from the ring C-2 but only glutamic acid derived from the α-carbon.

At least two steps are involved in the biological formation of ergothioneine from histidine: methylation of the amino nitrogen and attachment of sulphur to the C-2 of the imidazole ring. Thiolhistidine has never been shown to occur naturally and, moreover, synthetic L-2-thiolhistidine does not serve as a precursor of ergothioneine for *Neurospora crassa* (Melville *et al.* 1957). On the other hand, hercynin, the betaine of histidine, occurs naturally in certain fungi (Kutscher, 1910; Reuter, 1912) and would seem a likely intermediate in ergothioneine biosynthesis from histidine. If, in fact, this is so, methylation of histidine precedes sulphuration in the biosynthetic process.

SUMMARY

1. Cultures of *Claviceps purpurea* were grown on media containing either [α-^{14}C]histidine or

[2(ring)-^{14}C]histidine. Labelled ergothioneine was isolated from these cultures and chemically degraded.

2. The intact imidazole ring and side chain of histidine were shown to be incorporated into ergothioneine by this fungus.

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The Inhibition by γ-Irradiation of Incorporation of ^{32}P into Rat Thymocytes *in vitro*

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In a search for the primary biochemical lesions which are produced by ionizing radiation in animals, it would be convenient to have a mammalian system which is radiosensitive *in vitro* and which would provide sufficient material to permit a biochemical investigation. The inhibition of uptake of precursor into the nucleic acids of different tissues is a well-known consequence of irradiation *in vivo* (Euler & Hevesy, 1942) and can be detected very soon after the end of exposure (see Mitchell, 1956; Ord & Stocken, 1957). Howard & Pelc (1951), Lajtha, Oliver & Ellis (1954) and Totter (1954) have demonstrated the radiosensitivity of bean-root tips and bone-marrow cultures and suspensions to radiation *in vitro*, the inhibition of precursor uptake into nucleic acids being used as an index of radiation damage. Trowell (1952) found that cultures of lymph node are sensitive to exposure *in vitro*, and by using his cultures we have shown that incorporation of ^{32}P into their deoxyribonucleic acid is affected before histological damage can be detected (Ord & Stocken, 1956). On the other hand, the experiments on uptake of ^{32}P into the nucleic acids of thymus suspensions after exposure to 1000–2000 r. of γ-irradiation *in vitro* gave equivocal results (Ord & Stocken, 1956). The development of a medium suitable for the isolation of nuclei (Barnes, Esnouf & Stocken, 1957) prompted a re-examination of the behaviour of thymus suspensions in this medium, and they have now been found to be radiosensitive. It has therefore been possible to investigate some of the biochemical changes which accompany the inhibition of metabolism of thymus deoxyribonucleic acid.

METHODS

Animals. Young animals (70–130 g.) of the Laboratory stock of Wistar rats were used throughout. The results appeared to be independent of the sex and weight of the animal within this range.

Thymus suspensions. In the earlier experiments thymi from several rats were pressed through a stainless-steel gauze, suspended in a potassium-rich medium (0·0855 M-KCl, 0·0085 M-NaCl, 0·0025 M-$CaCl_2$, 0·0025 M-$MgCl_2$, 0·005 M-triethanolamine hydrochloric acid buffer, pH 7·2) supplemented with glucose (final concentration 10 mg./ml.) (Barnes *et al.* 1957) and filtered without squeezing through a single layer of muslin. This produced suspensions with not more than 30% of whole cells. To obtain 80–90% of undamaged cells, the capsule at the base of the thymus was sliced and the contents of the gland were gently scraped free. The operation was performed on a glass surface cooled with ice, and the resulting material was suspended in medium. From three to four rats 1 g. (wet wt.) of extruded thymocytes free from connective tissue was obtained, suspended in 4 ml. of medium and filtered as before. For irradiation and incorporation of ^{32}P, 1 ml. of suspension was incubated in Warburg flasks at 37°. It was thus possible to irradiate the cells while they were actively respiring and to measure the oxygen consumption during the experiment.

Cell counts. These were very kindly performed by Mr M. P. Esnouf by the differential-phase-contrast technique of Barer, Joseph & Esnouf (1956). Whole cells were distinguished from damaged and pycnotic cells and from nuclei. The results are given as total nuclei/ml. of suspension and as percentage of normal cells. Sufficient cells were counted to give an accuracy of ±5%.

Irradiation. Irradiation was provided by two 250 mc radium sources which were placed in Perspex holders beneath Warburg flasks (5 ml. capacity) containing 1 ml. of suspension (Ord & Stocken, 1955). With one source

beneath each vessel, a dose of 950 r. was received in 30 min. The dose was estimated by the ferrous sulphate-oxidation method (Miller, 1950).

Incorporation of ^{32}P. Carrier-free ^{32}P was obtained from the Radiochemical Centre, Amersham, Bucks. Unless otherwise stated, 25 μc was used/ml. of thymocyte suspension. In some experiments ^{32}P was given intramuscularly to the rats some hours before death, so that the release of ^{32}P from the organophosphates of thymus *in vitro* might be followed.

Inorganic phosphate. This was estimated by the method of Berenblum & Chain (1938).

Acid-soluble nucleotides. After incubation the flasks were chilled in ice and the protein was precipitated by 0·1 ml. of 100% (w/v) trichloroacetic acid. The suspensions were centrifuged and the supernatants stored at $-20°$ until required. The acid-soluble nucleotides were separated by paper chromatography by the technique of Krebs & Hems (1953), which allows removal of the inorganic phosphate. Usually 7 μl. of the supernatant was taken, but in some experiments samples from the concentrate obtained by evaporation from the frozen state were used. Inosine triphosphate, guanosine triphosphate, guanosine diphosphate, uridine triphosphate, uridine diphosphate, adenosine diphosphate (ADP) and chromatographically pure adenosine triphosphate (ATP) (Sigma Chemical Co., St Louis, Mo., U.S.A.) were used as markers. The nucleotides were located by ultraviolet photography.

Nucleic acid extraction. Deoxyribonucleic acid (DNA), ribonucleic acid (RNA) and deoxyribonucleotides were separated from the trichloroacetic acid precipitate as previously described (Deluca, Rossiter & Strickland, 1953; Richmond, Ord & Stocken, 1957). Our earlier experiments had demonstrated that incorporation of ^{32}P into DNA isolated by this technique reflected uptake of isotope into the deoxyribonucleotides. Blanks were run with thymus suspensions to which trichloroacetic acid was added before incubation with ^{32}P. Uptake into DNA under these conditions was negligible.

Measurement of radioactivity. ^{32}P activity in the inorganic phosphate, nucleic acids and nucleotides was measured in a Geiger–Müller liquid counter.

The location of the acid-soluble nucleotides on the chromatogram was detected by a radioautograph. The activity/unit area of paper was then measured by an end-window scanner. Sufficient counts were recorded to give an error of less than 3%.

RESULTS

Incorporation of ^{32}P *into nucleic acids.* In our earlier experiments it was suggested that the equivocal response to γ-irradiation shown by the rat-thymus preparations *in vitro* was due to structural damage to the cells which was detectable very shortly after their isolation (Ord & Stocken, 1956). The thymus suspensions in the high-potassium medium (Barnes *et al.* 1957) were therefore examined by phase-contrast microscopy initially and after 1 and 2 hr. of incubation at 37°. The appearance of the cells in the medium seemed normal and very little increase in pycnosis occurred during shaking for 2 hr. in the Warburg apparatus at 37°. Motility, however, was not used as an index of cell integrity.

In the first experiments (Table 1, group A) the suspensions were supplemented with 20 μmoles of succinate and 1 μmole of ATP, and were gassed for 2–3 min. with O_2 before incubation. In the first two experiments of the group, ^{32}P was incubated with the suspensions during the period of irradiation (30 or 60 min.) and the reaction was stopped as soon as the dose had been delivered. Since it may be presumed that incorporation starts with the addition of the isotope it is clear that a significant uptake will have occurred before irradiation can have produced a measurable effect. It was therefore decided for all subsequent experiments to irradiate the samples and to tip in the tracer as soon as the dose had been delivered. If both sources were present under one Warburg flask 950 r. were received in 15 min., and the procedure was therefore adopted of setting up paired vessels at 37°, one of which was irradiated. After 15 min. the sources were removed and ^{32}P was tipped into both suspensions. The incubations then continued for a further 30 min. period. It can be seen (Table 1, groups B and C) that the degree of inhibition depended on the dose received.

It was also noticed in the first group of experiments that if, under comparable conditions, the number of whole cells in the preparation was increased, uptake of ^{32}P into the nucleic acids also increased although the sensitivity of the incorporation was unaffected. This suggested that in this medium, uptake of precursor into DNA occurred only in the intact cells. This was supported by an experiment (Table 2) in which nuclei were prepared (Barnes *et al.* 1957) from a minced suspension, and uptake of ^{32}P into DNA and nuclear RNA of these nuclei was compared with that into DNA and RNA of a preparation of whole cells. Negligible incorporation occurred into DNA of the nuclei, although some uptake took place into nuclear RNA. This latter finding requires confirmation.

Since the sensitivity of the preparations in this first group of experiments was not great, it was decided to investigate the possibility of a protective effect due to the presence of the substrate (Dale, 1940). When succinate and ATP were omitted (Table 1, group B) it can be seen that uptake into DNA was reduced even more by irradiation, whereas that into RNA remained virtually unaffected. It was also established for a control suspension (Table 2) that the adjuvants did not alter the relative uptakes into RNA and DNA; all subsequent experiments were therefore performed without additions. Oxygen uptakes were always measured during the last 15 min. of incubation. Omission of succinate and ATP naturally reduced O_2 uptake, but in the large

Table 1. *Effect of γ-irradiation on the incorporation of ^{32}P into nucleic acids of rat-thymus suspensions*

Gas phase was oxygen unless otherwise indicated in parentheses in the first column. Suspensions of group A contained succinate and ATP (20 μmoles and 1 μmole respectively); those of groups B and C had no adjuvants. Dose: groups A and B, 950 r.; group C, 600 r.

	Cell counts		Specific activity (counts/min./μg. of P)				
			DNA		RNA	RNA/DNA	
	Total nuclei present (10^{-8} × no./ml.)	Percentage of normal cells	Control	Irradiated as % of control	Irradiated as % of control	Control	Irradiated
Group A	3·09	34	2·95	90*	110	8·15	9·93
	2·62	17	5·66	76·5†	115	7·35	11·1
	4·2	35	1·67	81	128	3·82	6·04
	5·02	88	12·3	85	107	3·55	4·47
	4·29	87	11·7	88	86	3·46	3·36
Mean (last three expts.)	4·50	—	—	85	107	3·61	4·82
Group B	4·35	84	4·34	64	99	3·1	4·8
	4·34	78	5·12	60·5	95	2·97	4·66
	3·51	78	3·02	81	98	4·62	5·60
	4·78	81	2·44	83	97	5·43	6·33
	3·61	87	3·7	64·5	93	3·65	5·25
	6·56	90	3·91	66	105	4·63	7·37
	4·3	83	2·46	80	102	4·21	5·39
	7·3	91	4·90	56·5	106	2·62	4·9
	7·1	91	4·51	78·5	125	2·54	4·05
	4·98	77	5·63	79·5	96	2·66	3·2
	4·18	87	6·49	74·5	106	2·5	3·57
	6·24	83	7·93	64	91	2·45	3·49
Mean	5·1	84	—	71	101	3·45	4·88
Group C	5·34	88·5	1·33	88	84	3·57	3·48
(Air)	5·34	88·5	1·69	83	100	3·54	4·26
	4·74	82	1·46	87	98	3·8	4·3
(Air)	4·74	82	1·97	91·5	91	3·8	3·98
	4·13	83·5	2·49	83·5	99	2·6	3·0
(Air)	4·13	83·5	3·36	84	120	2·56	3·68
Mean	4·74	85	—	86	99	3·31	3·78

* In the presence of ^{32}P (see text). † Dose 1900 r., in the presence of ^{32}P.

Table 2. *Incorporation of ^{32}P into nucleic acids of rat-thymus suspensions under different experimental conditions*

Details of these experiments are given in the text. The concentrations of succinate and ATP were as in Table 1.

	Cell count		Uptake of O_2 (μl./15 min.)	Specific activity (counts/min./μg. of P)		
	Total nuclei (10^{-8} × no./ml.)	Percentage of whole cells		DNA	RNA	RNA/DNA
Cells	3·96	80	31·6	11·1	25·0	2·25
Nuclei	5·17	10	5·0	0·67	9·33	—
Cells						
(*a*) With succinate and ATP	5·28	80	63	9·09	44·5	4·9
(*b*) Without succinate and ATP	5·28	80	46·6	1·92	9·8	5·1

number of experiments performed under standardized conditions without adjuvants, O_2 uptake was proportional to the total number of nuclei present in the preparation (whole cells, damaged cells, nuclei and free cytoplasm), and the O_2 uptakes did not differ significantly between the control and irradiated suspensions.

The last group (C) of experiments shown in Table 1 tested the effect of O_2 on the radiosensitivity of the preparation. Increased sensitivity in the presence of O_2 is a well-established factor in radiobiology (Gray, Conger, Ebert, Hornsey & Scott, 1953; Laser, 1956). Since anaerobiosis prevents the incorporation of ^{32}P into DNA (Findlay, Rossiter & Strickland, 1953) it was only practicable to compare the effects of irradiation on samples which had either O_2 or air as the gas phase during the experimental period. It can be seen (Table 1, group C) that the alteration in O_2 tension did not increase the sensitivity of the thymocytes but that flushing with O_2 appeared to reduce the incorporation of ^{32}P into both DNA and RNA; gassing was therefore discontinued in later experiments.

In experiments on the uptake of precursors into nucleic acids *in vitro* it was observed (Thompson, Smellie, Goutier & Davidson, 1956) that the ratio of incorporation into purine and pyrimidine deoxyribonucleotides was much less than that found *in vivo*. This was attributed to failure of the cells to synthesize purines at a sufficient rate, and it has been suggested (Davidson, Thompson, Paul, Smellie & Goutier, 1957) that it might be overcome in the intact rat by supplies from the liver. In our experiments, although a decreased uptake of ^{32}P into purine deoxyribonucleotides relative to values obtained *in vivo* (Ord & Stocken, 1956) was very evident (Table 3), irradiation still reduced the incorporation into all four deoxyribonucleotides.

Analysis of acid-soluble phosphates. The results shown in Table 1 indicated that γ-irradiation *in vitro* blocked uptake of ^{32}P into thymus DNA without affecting that into RNA. Perhaps the simplest explanation might be dilution of the inorganic phosphate pool in the irradiated samples by release of phosphorus from non-labelled compounds. This was investigated with suspensions of thymus which had been labelled with ^{32}P *in vivo*. An interpretation of the results of these leakage experiments was simplified by the absence of phosphate from the suspension medium. The concentration and specific activity of inorganic phosphate in trichloroacetic acid supernatants from these thymus suspensions after irradiation *in vitro* showed no difference in levels of either ^{31}P or ^{32}P between control and exposed preparations (Table 4). In the two experiments in which thymus nuclei were prepared and examined there was also no evidence for leakage of inorganic phosphate. This was confirmed in experiments (Table 5) in which uptake of ^{32}P again occurred *in vitro* after irradiation and the concentration and specific activity of the inorganic phosphate were measured, as well as the specific activity of the nucleic acids. Chromatographic separation of the acid-soluble nucleotides of the supernatant was also performed, and we are indebted to Dr G. V. R. Born for the estimation of the levels of ATP in the extracts by the firefly method (Strehler & Totter, 1952). It will be seen that again there were no consistent differences between the inorganic phosphate levels and specific activities of control and irradiated samples.

The chromatograms were run either with supernatants, used directly with ATP and other nucleotides as tracers, or with preparations which had been concentrated by evaporation from the frozen state. In the first method there was insufficient material in the absence of tracer to indicate the presence of the nucleotides by ultraviolet absorption, but the labelled components could easily be detected by radioautography. In the second method it was possible to demonstrate directly the presence of two main nucleotide components in the incubated samples, but neither method showed any qualitative differences between the components from control and irradiated suspensions. The faster-moving nucleotide was identified as ATP from its R_F and from its behaviour with tracer ATP. Only small amounts of ADP were detected in incubated samples and negligible activity was associated with it. The specific activity and amount of ATP was measured in preliminary experiments by elution of the nucleotide region on the chromatograms run with concentrated material. Since the amounts of ATP were low and the phosphorus blank was high, although the results showed a very marked increase in activity of the irradiated samples, an alternative method was finally adopted of locating by radioautograph the activity corresponding to ATP on the chromatograms, and then equal areas from

Table 3. *Relative specific activities of thymus deoxyribonucleotides* in vivo *and* in vitro

	Adenine	Guanine	Cytosine	Thymine
In vivo	0·71	0·93	0·57	1·0
In vitro	0·08	0·13	0·92	1·0
Effect of exposure to 950 r. *in vitro* (as % of control)	80	66	81	74

Table 4. *Effects of* 950 *r. of γ-irradiation on the release of inorganic phosphorus from thymus suspensions labelled* in vivo

Cell count		Phosphorus leak 15 min. after end of irradiation			
		^{31}P (μg./ml. of supernatant)		^{32}P (counts/min./μg. of P)	
Total nuclei (10^{-6} × no./ml.)	Percentage of whole cells	Control	Irradiated	Control	Irradiated
0·204	6·6	4·7	4·2	52	52
0·202	4·1	3·4	2·8	71	60
3·88	80	36·3	36·0	—	—
4·41	80	39·8	39·6	—	—
5·16	83·3	44·0	41·0	22·4	24·5
6·19	84·1	33·9	35·0	25·1	25·1
4·7	85·2	34·6	39·4	22·2	18·8

Table 5. *Concentration and specific activity of acid-soluble phosphates of thymus suspensions after* 950 *r. of γ-irradiation* in vitro

Concentrations are expressed as μg. of P/ml. and specific activities (S.A.) as counts/min./μg. of P. C, Control; R, irradiated suspension.

	Inorganic phosphate				ATP			Guanosine triphosphate S.A.	DNA S.A.		RNA S.A.	
	Concn.		10^{-4} S.A.		Concn.							
				100R		100R	S.A. 100R/C	100R		100R		100R
	C	R	C	C	C	C		C	C	C	C	C
	33·9	35·0	9·3	98	—	—	138	—	3·52	66	16·3	105
	28·0	31·8	14·7	90	—	—	125	108·5	5·75	78·5	14·6	125
	29·9	29·6	14·3	101	—	—	100·5	105·5	6·0	56·5	15·7	106
	32·3	31·1	12·4	98	—	—	122	105·5	3·78	79·6	15·9	102
	27·6	30	12·2	93·5	11·1	96·5	101	100	5·63	79·5	14·95	96
	30·1	28·1	11·2	108	12·0	110	113·5	108	6·49	74·5	16·2	106
	34·8	35·6	9·5	100	13·2	108	107	99	7·95	64	19·4	91
Mean	30·9	31·6	—	98	—	105	115	104	—	71	—	104

control and irradiated suspensions were counted with an end-window scanner. For this purpose the trichloroacetic acid supernatants were not concentrated, and equal amounts were taken from each sample. The samples were run at least in duplicate. It can be seen that there was an increase in ^{32}P activity of ATP from the irradiated suspensions.

The other nucleotide on the chromatograms had a considerably smaller R_F than ATP, and intervening between these regions there were at least two major components which were not nucleotides. These were thought to be sugar phosphates but were not further examined. The slower-moving nucleotide was identified as guanosine triphosphate from its ultraviolet absorption (Beaven, Holiday & Johnson, 1955), and from its behaviour on chromatograms run with guanosine tri- and di-phosphate as tracers.

DISCUSSION

In these experiments it has been possible to obtain reproducible inhibition of incorporation of ^{32}P into the DNA of rat-thymus suspensions by irradiation *in vitro*. We have not investigated in detail media other than the one used here, so that it is not possible to say whether these effects might also be produced with nuclei rather than with whole cells. Allfrey, Mirsky & Osawa (1955, 1957) have shown, in a sucrose medium, that isolated thymus nuclei are metabolically active especially in protein synthesis and the intranuclear generation of ATP. They also reported that other media tried were not suitable for demonstrating this activity, which may explain the failure of thymus nuclei in our experiments to incorporate ^{32}P.

The radiosensitivity of the incorporation was decreased by the presence of succinate and ATP in the medium, and experiments demonstrated that the ATP was very rapidly hydrolysed, probably to the adenosine monophosphate level. Protection of cell suspensions against irradiation by oxidizable substrates is well established, particularly with micro-organisms (cf. Goucher, Kamei & Kocholaty, 1956).

Lajtha, Oliver, Kumatori & Ellis (1958) have shown with bone-marrow preparations *in vitro* that the synthetic period (*S*) in interphase is less sensitive than the G_1 period immediately preceding it. They concluded that 'There is a system connected with, but not identical to, DNA synthesis which is more sensitive than the process of synthesis itself.' They did not investigate RNA or

other cytoplasmic components in any detail. Since ^{32}P was incubated with our suspensions for only 30 min. immediately after exposure, if the timing of the mitotic cycle in thymocytes is comparable with that in bone marrow then the effects on DNA must have been predominantly with cells in the *S* period. Their sensitivity appears to be similar to that reported by Lajtha *et al.* (1958).

The lack of effect on RNA resembles results obtained for incorporation of ^{32}P with lymph-node cultures (Ord & Stocken, 1956), where inhibition of uptake of ^{32}P into DNA was obtained 2 hr. after exposure to 600 r. *in vitro*, with no effect on RNA and no histological signs of damage to the cells. In thymus, by 30 min. after 1000 r. of total body irradiation, nuclear and cytoplasmic RNA are affected as well as DNA (Ord & Stocken, 1957), but in liver, although ^{32}P uptake into DNA is reduced, that into RNA is unaffected or increased (Sherman & Almeida, 1957; Richmond *et al.* 1957). It is not yet possible to say whether the effect on RNA metabolism *in vivo* occurs as a result of damage to the DNA system, the whole process proceeding more rapidly than with isolated preparations, or whether the suspensions and cultures used *in vitro* may have lost that part of the radiosensitive mechanism which causes reduction in RNA metabolism *in vivo*. A further consideration is that isolated preparations are not exposed to secondary changes which occur in the intact animal as a result of exposure (Holmes, 1949; Harrington & Lavik, 1957). Whatever the explanation for the results *in vivo* it is clear that in these experiments *in vitro* incorporation of ^{32}P into DNA was reduced without alteration of RNA, and this was accompanied by an increased turnover of ^{32}P into ATP. Mitchell (1942) reported the accumulation of a nucleotide-like material in the cytoplasm of cells after irradiation and suggested that exposure produced a block between RNA and DNA. Sherman & Forssberg (1954) and Forssberg & Klein (1954) found transient alterations in the levels and specific activities of adenine polynucleotides in liver and ascites-tumour cells immediately after exposure *in vivo*; in mouse liver the specific activity of ATP was increased 5 min. after exposure. In our experiments with rat thymus we could detect no change in incorporation of ^{32}P into acid-soluble nucleotides 1 and 2 hr. after exposure to 1000 r. *in vivo*, but the nucleotides were estimated by chemical means and not by the more accurate chromatographic separation. Since in the experiments *in vitro* uptake of ^{32}P into RNA was not inhibited, it appears either that the machinery for synthesis of ribonucleotides is unaffected by γ-irradiation or that there is an adequate reserve of utilizable precursors for the duration of the experiment. The increase in specific activity of ATP suggests either that the ATP is not available to the nucleus for DNA synthesis or that there is block in the generation of deoxyribonucleotides (cf. Mitchell, 1942). It may be significant that enucleation in amoebae causes an accumulation of ATP in the cytoplasm under aerobic conditions (Brachet, 1955, 1956).

SUMMARY

1. A medium has been found in which a large proportion of a respiring suspension of thymus cells retain their normal appearance, as judged by phase-contrast microscopy, for periods up to 2 hr.

2. The uptake of ^{32}P into the deoxyribonucleic acid of the respiring thymocytes was inhibited by 600–950 r. of γ-irradiation.

3. There was an increase in the specific activity of adenosine triphosphate in the irradiated suspensions.

4. Incorporation of ^{32}P into ribonucleic acid was unaffected *in vitro*.

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The Chemical Composition of Earthworm Cuticle

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Since the work of Astbury (1938) and Bear (1952) it has become usual to define collagen as a group of fibrous proteins which give a characteristic wide-angle X-ray-diffraction pattern, and collagens defined in this sense have been found widely distributed throughout the animal kingdom (Bear, 1952; Rudall, 1955). That a large number of different proteins have identity of wide-angle X-ray-diffraction pattern implies that these proteins have certain features of their molecular structure in common, and the present definition therefore includes all those proteins in which these features are present.

The highly characteristic amino acid composition of collagens derived from mammalian sources, and especially the fact that in many analyses the glycyl residues appear to be exactly one-third of the total number of residues present, has provoked much comment, and indeed in some models is a prerequisite of the stereochemical packing of the polypeptide chains. It is, in this context, interesting to explore the range of chemical composition which is possible within the framework of the collagen structure. Mammalian collagens form a compact group with regard to their amino acid compositions (Eastoe, 1955; Neuman, 1949), and therefore for this purpose it is necessary to examine collagens from a wider range of animals.

Earthworm cuticle has been shown to give the wide-angle X-ray pattern of collagen (Astbury, 1938; Rudall, 1950) and is readily obtained in a morphologically pure state for chemical analysis. A preliminary account of this work has been published (Watson & Smith, 1956).

EXPERIMENTAL

Isolation of earthworm cuticles. Batches of 100 earthworms (*Lumbricus* sp.) were killed by drowning them in ether. After immersion for 15 min. the cuticles may be readily removed by rolling them back from the anterior end with forceps. The cuticles were then washed with numerous changes of water in order to remove adhering columnar epithelial cells. In the first two preparations this was followed by incubation with 0·1% trypsin in tap water at room temperature for 8 hr. The cuticles were finally washed in water. For the determination of the hydrothermal-shrinkage temperature the cuticles were used without further treatment. For chemical analysis the cuticles were dehydrated with two changes of absolute ethanol and two changes of ether, and were allowed to dry in air. At this point the cuticles should appear quite white and any yellow coloration may be taken as an indication of the presence of epithelial material, in which case the washings should be repeated. The yield of air-dry cuticle was usually about 300 mg. The cuticles were then cut into small pieces with scissors, and mixed thoroughly. The samples were allowed to equilibrate for 48 hr. with the air of the laboratory before weighing.

Moisture and total nitrogen. The determinations were performed according to Chibnall, Rees & Williams (1943).

Determination of amino acids. Samples (50 mg.) of cuticle were hydrolysed in sealed tubes with 5 ml. of 6N-HCl for 24 or 40 hr. The temperature was controlled by immersing the tubes in a glycerine bath at 110°. The hydrolysates were fractionated on columns of Dowex-50 by the technique of Moore & Stein (1951) with the modifications suggested by Eastoe (1955). The length of the small column was increased from 15 to 20 cm. The fractions (1 ml.) were analysed by the photometric method of Moore & Stein (1954), 1 ml. of the reagent being added to each fraction.

Determination of imino acids. Hydroxyproline was estimated by the method of Neuman & Logan (1950). Proline could not be estimated by integrating the peak on the amino acid chromatograms since it was so small. Proline was therefore estimated by the more sensitive colorimetric method of Chinard (1952), after separation of interfering amino acids on a short column of Dowex-50 according to Rogers, Kimmel, Hutchin & Harper (1954). The rate of flow of the column was decreased to 2 ml./hr. to sharpen the proline peak. Fractions (1·7 ml.) were collected and heated with 2 ml. of acetic acid and 2 ml. of the ninhydrin reagent (Chinard, 1952). The solutions were diluted to 10 ml. with acetic acid before readings of optical density were made.

Identification of sugars present. Earthworm cuticle (50 mg.) was hydrolysed with N-H_2SO_4 in a sealed tube at 110° for 16 hr. The hydrolysate was deproteinized and freed from amino acids and peptides by the procedure of Gottschalk & Ada (1956). The hydrolysate was finally freeze-dried, taken up in 0·5 ml. of water and 10 μl. portions were taken for paper chromatography. The chromatograms were developed for 48 hr. by the descending technique. The solvents used were *n*-butanol–ethanol–water (40:10:50) (Hirst & Jones, 1949), and *n*-butanol–pyridine–water (50:30:20) (Chargaff, Levine & Green, 1948).

Estimation of hexose. The analyses were performed upon a portion of the N-H_2SO_4 hydrolysate taken before deproteinization by the anthrone method of Loewus (1952), galactose being used for the construction of standard curves.

Estimation of hexosamine. The direct estimation of glucosamine and galactosamine from the peaks on the amino acid chromatograms as suggested by Eastoe (1954) was not applicable, since variable results were obtained from one hydrolysate to another. A higher and more reproducible result was obtained after hydrolysis of the cuticle at 110° with 5N-HCl, by the method of Elson & Morgan (1933) as modified by Kelly (1956).

Estimation of pentose. Pentose was estimated by the method of Mejbaum (1939) as modified by Albaum & Umbreit (1947). Arabinose was used for the preparation of standard curves.

Estimation of amide nitrogen. Three 100 mg. samples of cuticle were analysed by the method of Bailey (1937).

RESULTS

Earthworm cuticles when freshly isolated in water are transparent membranes with a hydrothermal-shrinkage temperature of 45°. The bulk of the cuticle passed into solution in water at temperatures above 45°. Air-dried cuticle (50 mg.) was extracted with 1 ml. of water in a stoppered test tube at 100° until most of the cuticle had passed into solution. This solution set to a gel upon cooling to 4°.

Earthworm cuticles have a low ash content (0·14 %), are insoluble in dilute hydrochloric acid but more soluble in dilute solutions of sodium hydroxide, and most of the cuticle will dissolve in cold 2M-potassium thiocyanate.

Estimations of total nitrogen and hydroxyproline have been carried through on each of the seven preparations used during the course of this investigation. The total nitrogen contents of all preparations were between 14·43 and 14·82 %, the mean value being 14·61 %, with a standard deviation of ±0·16. The hydroxyproline contents of the preparations were between 17·3 and 17·8 %, the mean value being 17·5 %, with a standard deviation of ±0·2. When earthworm cuticle was hydrolysed for the determination of the amino acids about 3 % of insoluble humin was formed. This was centrifuged down and analyses were carried through on the supernatant. Three preparations have been subjected to amino acid analysis and were found to be indistinguishable, within the limit of accuracy of the colorimetric technique (±2 %). The analyses are reported in Table 1.

Corrections of +13 and +4 % to serine and threonine respectively have been made for decomposition during hydrolysis for 24 hr., based on a comparison of the yields of serine and threonine which were obtained from 24 and 40 hr. hydrolysates of earthworm cuticle.

Examination of earthworm-cuticle hydrolysates for sugars by paper chromatography gave evidence of five components, of which one only was present in high concentration. This principal sugar moved at the same rate as a galactose marker which was run in parallel; glucose did not appear to be present.

Extraction of cuticle with water. When earthworm cuticle was treated with water at temperatures above its shrinkage temperature the bulk of the cuticle passed into solution. However, some residue was always left.

Air-dried cuticle (equivalent to 85·1 mg. of dry cuticle) was extracted at 100° for 3 min. with 10 ml. of water. The residue was re-extracted twice with 5 ml. of water under the same conditions. The residue was then washed with absolute ethanol and ether and allowed to dry in air. The yield was 18·5 mg. dry wt. The material (N, 12·5 %; hexose, 15·4 %) appeared to contain a greater proportion of polysaccharide than the original cuticle.

The combined extracts were treated with one drop of M-sodium acetate and 2 vol. of absolute ethanol and allowed to stand overnight. The precipitate was centrifuged down, washed with

Table 1. *Amino acid composition of earthworm cuticle and the gelatin derived from it*

	Earthworm cuticle			Gelatin fraction			Ox-hide collagen (Bowes, Elliott & Moss, 1955)
	g./100 g.	m-moles/g.	N as % of total N	g./100 g.	m-moles/g.	N as % of total N	N as % of total N
Total nitrogen	14·61	—	—	15·9	—	—	—
Glycine	19·6	2·61	25·0	22·8	3·04	26·8	26·6
Alanine	7·72	0·866	8·31	8·67	0·973	8·57	8·72
Leucine	3·74	0·285	2·73	3·52	0·268	2·36	2·14
Isoleucine	1·94	0·148	1·42	1·89	0·144	1·27	1·08
Valine	2·15	0·184	1·77	1·88	0·161	1·42	1·58
Phenylalanine	1·92	0·116	1·11	0·81	0·049	0·43	1·07
Tyrosine	0·63	0·035	0·33	0·0	0·00	0·0	0·41
*Serine	9·3	0·89	8·5	10·4	0·99	8·7	3·06
*Threonine	6·2	0·52	5·0	5·8	0·49	4·3	1·43
Cystine	0·0	0·00	0·0	0·0	0·00	0·0	—
Methionine	0·0	0·00	0·0	0·0	0·00	0·0	0·49
Proline	1·41	0·123	1·17	—	—	—	9·43
Hydroxyproline	17·5	1·34	12·8	20·4	1·56	13·7	7·37
Arginine	3·21	0·185	7·04	3·47	0·199	7·02	14·22
Histidine	0·08	0·005	0·15	0·0	0·0	0·0	1·02
Hydroxylysine	0·0	0·00	0·0	0·0	0·00	0·0	1·07
Lysine	1·95	0·133	2·58	2·02	0·138	2·43	4·08
Aspartic acid	6·88	0·517	4·95	6·99	0·526	4·62	3·93
Glutamic acid	10·17	0·691	6·63	11·25	0·765	6·73	5·69
Amide nitrogen	1·200	0·856	8·21	1·28	0·913	8·1	3·50
Hexosamine	1·76	0·098	0·94	0·88	0·049	0·043	—
Hexose	14·0	0·78	—	10·6	0·59	—	—
Pentose	2·4	0·16	—	—	—	—	—
Total	—	†8·66	98·7	—	†9·30	96·9	—

* Corrected for decomposition during hydrolysis (see text).
† Not including amide, hexosamine, hexose or pentose.

absolute ethanol and ether and allowed to dry in air. The yield was 58·2 mg. dry wt. This fraction, the gelatin fraction, contained rather less polysaccharide than the original cuticle (Table 1). This gelatin fraction was submitted to amino acid analysis, and the results are included in Table 1. Two analyses were performed, one on a 40 hr. and the other on a 24 hr. hydrolysate, the results being concordant. There was not enough material left for a separate determination of proline and amide nitrogen, and the latter was estimated by integration of the ammonia peak from the 20 cm. column, followed by correction for the ammonia produced by the decomposition of serine and threonine during hydrolysis (Eastoe, 1955).

DISCUSSION

The protein component of earthworm cuticle is associated with a very much larger amount of polysaccharide material than is found in the vertebrate collagens. The same is true of two morphologically distinct types of fibril from the mesogloea of the sponge *Spongia graminea*, recently analysed by Gross, Sokal & Rougvie (1956). The similarity extends further. Upon hydrolysis of these three materials the amount of hexoses liberated is much greater than the amounts of pentose and hexosamine. These are the only collagenous materials from invertebrate animals for which polysaccharide analyses are available, but they would seem to indicate that the relationship between the protein components (indicated by the similarities in the wide-angle X-ray patterns and the presence of hydroxyproline in both materials) extends at least to a superficial similarity in composition between the polysaccharide components.

It will be noticed that earthworm cuticle contains a large proportion of glycyl residues, amounting to approximately 30 residues for every 100 residues present. This proportion rises in the gelatin fraction to about 32 glycyl residues in every 100 residues. A similar relationship between vertebrate collagens and gelatins has been observed by Eastoe (1955), and it was suggested that small amounts of non-collagenous proteins containing low proportions of glycine had been removed during the production of the gelatin. This conclusion was also compatible with the remainder of the amino acid composition of gelatin, and is also

true of the data presented for earthworm cuticle and gelatin in Table 1.

Since technical improvements in the X-ray-diffraction photography of collagen in this laboratory (Cowan, North & Randall, 1953) it has been recognized that the polypeptide chains in collagen are disposed in a helical structure, and several models based on this principle were subsequently proposed. The models which have provided the closest fit to the X-ray-diffraction patterns and infrared data employ a coiled-coil structure of three polypeptide chains (Cowan, McGavin & North, 1955; Ramachandran & Kartha, 1955; Rich & Crick, 1955). Some of the models described in these publications require that every third residue in each polypeptide chain be a glycyl residue, for stereochemical reasons. Although no evidence for the sequence of the residues in earthworm cuticle has been obtained, it is thought that the amino acid analysis of earthworm cuticle gives circumstantial analytical evidence of this requirement.

Earthworm cuticle has been shown to lack some of the main distinguishing features of a mammalian collagen fibril. Reed & Rudall (1948) report that the material does not show the familiar 640 Å banding visible in electron micrographs of collagen fibrils, and this observation has been confirmed by Dr S. Fitton Jackson in this Laboratory. Also it has been shown above that the protein component of earthworm cuticle shows no trace of the amino acid, hydroxylysine, an amino acid which is found only in hydrolysates of vertebrate collagens. It may also be noted that earthworm cuticle differs from vertebrate collagens in that the number of free acidic groups may just exceed the number of free basic groups (Table 2). Earthworm cuticle also belongs to a growing number of collagenous proteins [(Damodaran, Sivaraman & Dhavalikar (1956), Eastoe (1957)] which do not fit into the correlation between hydroxyproline content and hydrothermal-shrinkage temperature observed by Gustavson (1955) in a variety of fish collagens. It now seems unlikely that bonds involving specifically the hydroxyl group of hydroxyproline have a great stabilizing effect upon all collagen fibrils.

Very recently Singleton (1957) has published some analyses of earthworm cuticles isolated from *Allolobophora longa*. In detail these analyses are rather different from those reported above. The *Allolobophora* cuticles appear to contain less polysaccharide than do the *Lumbricus* cuticles, and some of the amino acid values are rather different. It is not clear whether this represents a species difference or is a result of the extensive pretreatment given to the *Lumbricus* cuticles.

Table 2. *Comparison of charged side chains in earthworm cuticle with those in ox-hide collagen*

Values are given as m-moles/g.

	Earthworm cuticle	Ox-hide collagen (Bowes, Elliott & Moss, 1955)
(*a*) Aspartic acid	0·517	0·522
(*b*) Glutamic acid	0·691	0·756
(*c*) Amide	0·856	0·465
Acidic side chains ($a+b-c$)	0·352	0·813
(*d*) Arginine	0·185	0·472
(*e*) Histidine	0·005	0·045
(*f*) Lysine	0·133	0·271
(*g*) Hydroxylysine	0·00	0·070
Basic side chains	0·323	0·858

SUMMARY

1. Earthworm cuticles were isolated from *Lumbricus* sp. in a form which yielded reproducible chemical analyses.

2. The analyses show that approximately 80% of the cuticle consists of a protein component, which contains a high proportion of hydroxyprolyl and glycyl residues, but not hydroxylysyl residues.

3. The remainder of the cuticle is chiefly composed of polysaccharide, which when hydrolysed yields galactose and small amounts of both pentoses and hexosamines.

4. A gelatin fraction was isolated from the cuticle and submitted to chemical analysis.

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Freeze-Drying Equipment for Large-Scale Laboratory Use

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The principles and practice of the freeze-drying process have been reviewed by Greaves (1946) with special reference to the large-scale drying of human serum and plasma, and the field has been surveyed in detail by Flosdorf (1949). More recently these authors and others have contributed authoritative articles on the subject (see Harris, 1954). Simple apparatus for the freeze-drying of small quantities in the laboratory has been described by various workers, e.g. Greaves (1956). However, for laboratory freeze-drying on the large scale more elaborate apparatus is desirable, and it was to meet such a demand that the equipment described in this paper was designed. Except for certain ancillary items such as vacuum pumps and compressors, the construction of the machines has been carried out entirely in our engineering section. The main aim in design was to provide a freeze-drying service which could be operated without any special skill, with the minimum of attention and handling of material, and which would provide a satisfactory end-product whether in small ampoules or large bulk volumes of 10 l. or more. The equipment has given good service in this Establishment with the minimum of trouble over a number of years.

The three types of machine described all operate on the vacuum spin–freeze principle introduced by Greaves (1944), in which an aqueous solution is caused to freeze rapidly by its evaporation in the vacuum chamber in which it is subsequently dried, any foaming or frothing of the material during freezing being suppressed by centrifugal force. Various proprietary makes of laboratory freeze-driers make use of this principle by placing the small ampoules or bottles in inclined holes in a rotating plate, so that the material freezes as a wedge of varying thickness at one side of the ampoule. It is clear that the extension of this method to deal with greater volumes of material contained in larger vessels would result in a wedge of material of considerable thickness with a proportional increase in drying time such as to render the method too slow to be practicable. Thus with the larger size of container it becomes imperative to spin each vessel in the vacuum chamber about its longitudinal axis at a speed sufficient to distribute the contents in a uniformly thick layer around the walls. Although more elaborate equipment is required to achieve this end, certain advantages are gained over the usual methods involving prefreezing. Prefreezing in a blast of cold air is a slow process which produces ice crystals of large size and an unsatisfactory dried product that is difficult to reconstitute. Even when the liquid is clean enough to allow supercooling to take place one has often to reckon with the possibly deleterious effects of exposure of proteins, etc., to increasingly high salt concentrations for long periods during the later stages of freezing. Freezing can, of course, be completed more rapidly by rotation of the container, especially if it is of thin glass, in a low-temperature bath but it may then become necessary to wipe clean the outside of the con-

tainer before loading into the vacuum chamber in order to avoid contamination of the pump oil. There is also the danger, should there be any tendency to supercooling, of sudden freezing of the mass on one side of the vessel. With either method, and especially with small bottles, rapid loading into the freeze-drying chamber is essential in order to establish the vacuum before any thawing of the material has had time to occur.

Satisfactory evaporative freezing depends upon the following conditions:

(1) The production of sufficient centrifugal force by rotation of each container about its longitudinal axis in the vacuum chamber to ensure (*a*) suppression of foaming, which according to Dr R. I. N. Greaves (see Harris, 1954) requires only about 7 ***g***., and (*b*) distribution of the contents as a uniformly thick layer, the magnitude of the force required then depending on the height of the container.

(2) A vacuum pump of sufficient capacity in relation to the size of the vacuum chamber to reduce the pressure from a few mm. Hg, when freezing commences, to about 0·1 mm. (corresponding to the vapour pressure of ice at the coil temperature) within a few minutes.

(3) A compressor capable of dealing at $-30°$ or lower with the rapid evaporation and condensation of water vapour in a quantity sufficient to cool the liquid (and container) to 0°, to remove the latent heat of fusion and to cool the ice so formed to $-25°$ or lower, i.e. at least 110 cal./g. of water to be dried. During this stage, the evaporation and condensation of about one-sixth of the total amount of water to be dried must therefore take place in as short a time as possible and the compressor is here called upon to deliver its maximum duty. If adequate duty is not available, freezing will be unduly prolonged. The demand could be met by circulating cooled methanol from a large refrigerated storage tank, but we have preferred to use large compressors which then run 'light' during the remainder of the freeze-drying procedure.

TYPES OF MACHINE

To meet the demand for a wide variation in the volume of material to be freeze-dried, it has been found desirable to construct three different types of machine, each designed for spinning the individual containers in the vacuum chamber. The drive is obtained by gear wheels throughout in order to avoid any risk of slip, but to simplify manufacture they are of the spur type, noise and wear being reduced by constructing some of the gears of Tufnol (Tufnol Ltd., Perry Barr, Birmingham). The main drive is supplied via a central shaft, through a special vacuum seal, from a motor mounted beneath the chamber. The refrigerated coil is located in the lower part of the chamber, and presents a sufficiently large surface to accommodate, as a fairly thin layer, the maximum quantity of ice likely to be condensed upon it during the drying of a batch of material. Fig. 1 shows a typical layout of one of the machines (Type 2) with control panel. The compressor and vacuum pump are housed in a sound-proof compartment behind the panel.

Type 1 machine designed to accommodate 60 of the 1 oz. (30 ml.) screw-cap type of bottle or the 1 oz. ampoule

This has already been briefly described and illustrated (Record & Taylor, 1953). Each of the sixty bottle carriers (int. diam., $1\frac{1}{8}$ in.) is machined in one piece from stainless-steel rod and runs in two ball bearings located in the carrier head. This head is readily removed by withdrawing six studs and can be interchanged with a solid rotor for use with smaller ampoules for drying in the wedge form. A steel gear on the central driving shaft meshes with each of six bronze gear wheels on the inner ring of carriers and each one of these drives the remaining nine (Tufnol) gear wheels in its corresponding sector. The first and last members of the gear train in each sector are in mesh with two of their neighbours, there being ample backlash in the intermediate gears. The Monel tubular heating element located beneath the carrier head can be loaded to a maximum of 0·5 kw, control being effected by a Variac transformer, and a chromium-plated reflector throws the heat upwards to metal disks on the gear wheels whence it is conducted through to the carriers. A small sealed thermostat, which is pre-set to switch off at 30°, to prevent overheating the product, is inserted in a hole situated in the side of the carrier head. The central shaft is gear-driven by a 0·5 h.p. motor to give a final carrier speed of 2000 rev./min., which is more than adequate in suppressing foaming and gives ample centrifugal force to distribute the liquid uniformly round the walls of the small containers used. The vacuum seal on the central shaft is of similar design

Fig. 1. A general view of one of the machines with control panel.

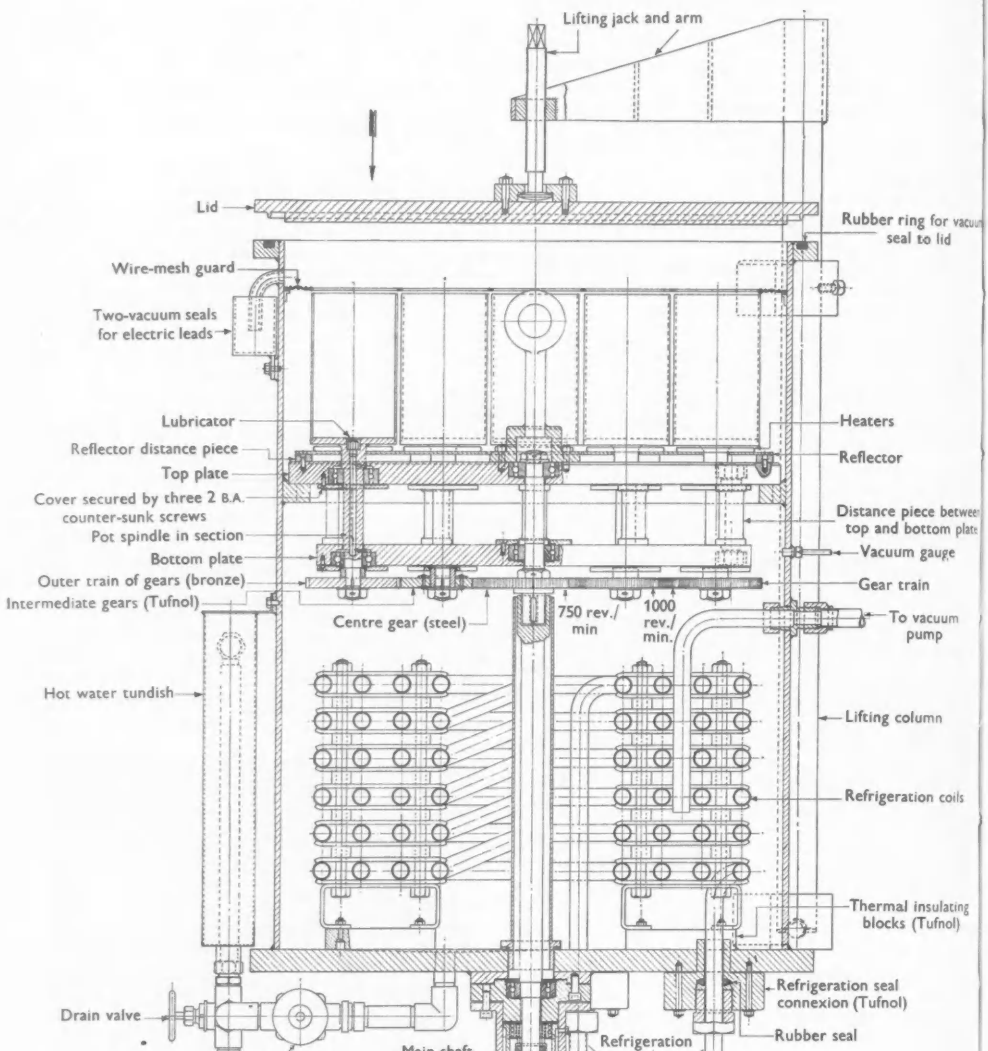

Fig. 2. General arrangement of type 2 machine in section.

and construction on all three types of machine, and is described in detail below. The condensing coil is maintained at about −45° by a 1 h.p. water-cooled compressor with Freon 12 as refrigerant, and the vacuum is obtained by the use of a two-stage rotary oil pump of 150 l./min. displacement.

The volume of liquid in a 1 oz. bottle is restricted to 15 ml. since loss of liquid past the shoulder of the bottle is likely to occur if this is exceeded. Although this loss is not a problem with 1 oz. ampoules, the same limit of volume is observed to minimize the risk of cracking the glass during the freezing stage. As the 1 oz. bottles taper slightly towards the base, a thin rubber band round this portion helps to centre the bottle.

When operating the machine, spinning is necessary for the first 20–30 min. only, by which time the vacuum in the chamber has reached 0·1 mm. Hg and the product temperature is −25°. With the thin liquid layer, freezing takes place very rapidly, there being no discontinuity in the recorded product temperature to show when freezing actually occurs. Throughout the main part of the drying procedure the product assumes a temperature of about −40° with open-necked bottles, or about −35° when aseptic precautions are taken by the fitting of sterile-filter caps. With 15 ml. of water/bottle, i.e. a total load of 0·9 l., drying is complete in about 6 hr. without the application of heat. Two batches, i.e. 120 bottles or 1·8 l., can be conveniently processed every 24 hr. on this machine, with overnight running.

Type 2 machine to accommodate 18 fruit-bottling jars

In selecting a container suitable for the freeze-drying of moderate quantities of material, the standard fruit-bottling jar with glass lid, rubber sealing ring and screwed-metal closure has much to commend it, since such bottles have proportions ideally suited to freeze-drying, are cheap and readily obtainable, are made to close tolerances in size, withstand moderate temperature changes and are fairly robust mechanically. The glass lid with narrow rubber-ring seal offers advantages for storage of the dried material in having the minimum amount of rubber through which diffusion of water vapour can occur. The 3 lb. size (capacity approx. 1 l.) was chosen as the most suitable compromise for our present requirements.

The general arrangement of the machine is shown in Fig. 2. The vacuum chamber is of welded construction and was rolled from ¼ in. mild-steel plate, the base and lid being of ⅞ in. steel. The chamber is opened by raising the lid by means of the screw jack and swinging it to one side. The bottle carriers are made of drawn-steel tube (int. diam., 4⅛ in.) which allows for the fitting of thin rubber-ring cushions around the top and bottom of each jar. The tubes are welded to a steel base and spigot, and chromium-plated on the outside, except for the underside which has a matt-black finish. A 1 h.p. motor drives the central shaft, which enters the chamber through a vacuum seal. At the upper end the shaft terminates as a keyed socket which engages a steel spur gear driving the inner ring of six Tufnol gears, each of these driving two bronze gears in the outer ring. The complete carrier and gear assembly may be removed from the chamber by withdrawing six bolts. This part of the machine is shown in Fig. 3. The condensing coil is cooled by the direct expansion of Freon 12, a 4 h.p. water-cooled compressor being used, and the vacuum line is connected to a two-stage rotary oil pump of 150 l./min. displacement. Fig. 4 is a view of the inside of the open chamber showing some filter-capped jars in position. The thermocouple pickup on one of the jars is also shown.

Heat is applied during drying by a circular disk-type heater (with a maximum loading of 125w each) situated under each carrier, all the eighteen heaters being connected in parallel and controlled by a Variac. An earlier model had provision for switching into circuit only those heaters actually required, but conduction of heat through the carrier head makes this unecessary. With the varied load of material, both in respect of quantity per jar and nature of material, usually dealt with in this machine the use of a large heat input is precluded and it is then advisable to use only a nominal heat input of about 200w, which raises the final temperature of the dried material to about 35°. With a uniform load this heat input could be

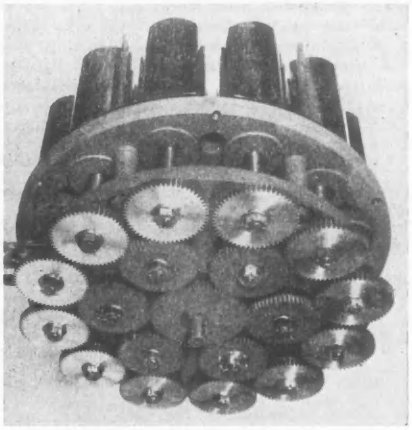

Fig. 3. Type 2 machine: carrier head and gear assembly

Fig. 4. Type 2 machine: view from above. Six filter-capped jars are shown in position.

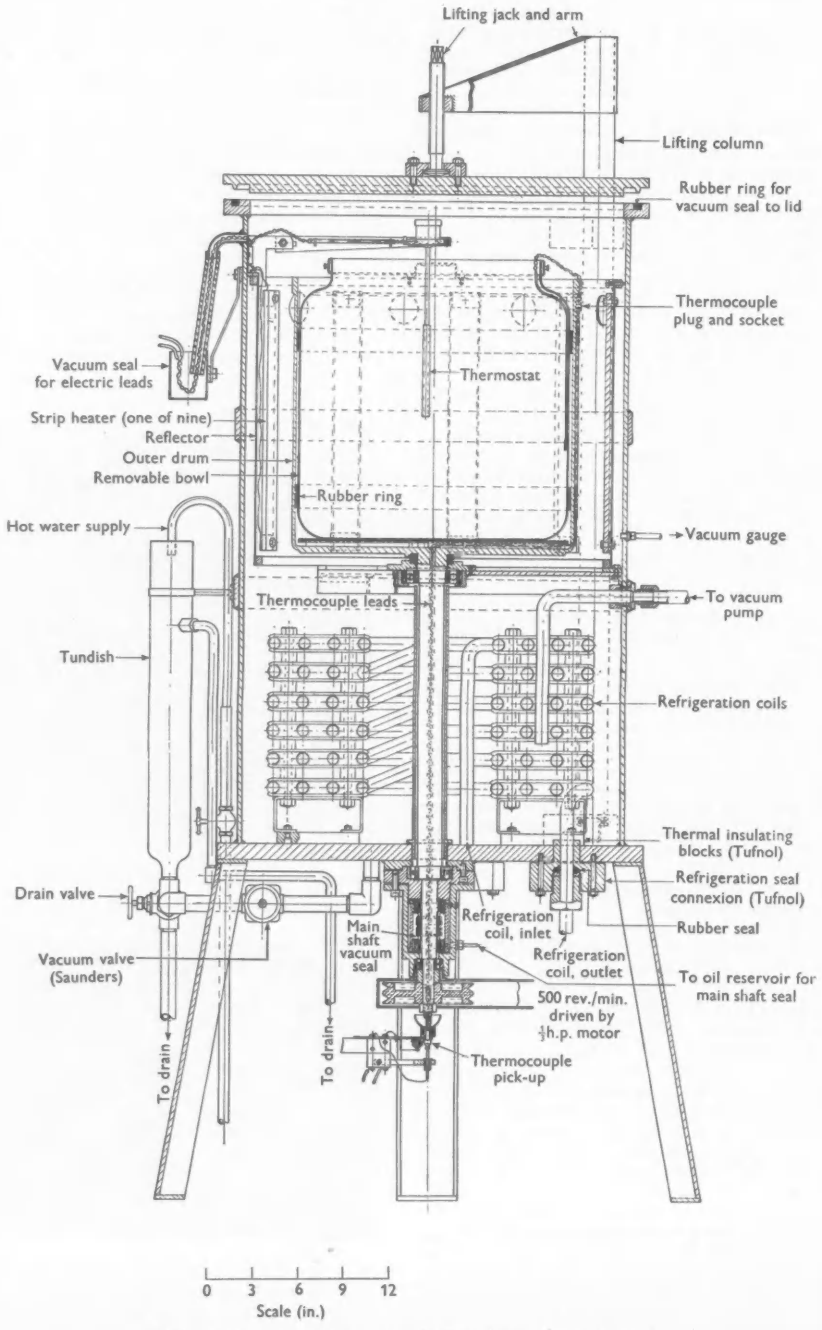

Fig. 5. Type 3 machine: sectional view of general arrangement.

greatly exceeded, provided that an automatic device was incorporated to switch off the heat as the product approached dryness. However, with the low heat input specified, and a load of 200 ml./jar (a total load of 3·6 l.), a comfortable margin of time is allowed for a 24 hr. turnover, and any risk of cracked jars is avoided.

The jars are spun at 1000 rev./min., which produces a centrifugal force on the surface of a $\frac{1}{4}$ in. thick layer of liquid (200 ml.) of about 45 *g*. This far exceeds the force necessary to suppress foaming but is required in order to produce a uniformly thick layer of liquid round the wall of the jar. The inner layer surface will then be inclined to the vertical by about 1 in 45, corresponding to a gradation in thickness of about $\frac{1}{8}$ in. over the 6 in. height of the liquid layer. Where it is necessary to handle the material aseptically, the metal-screw caps of the jars can be fitted with filters, consisting of a cotton-wool pad sandwiched between layers of muslin, and wired to the metal-screw caps. This presents a slight obstruction to the flow of water vapour from the jar, and raises the drying temperature by about 5° under normal conditions (200 w heat input).

The bottling-jar type of closure is well suited for storage of the dried material, but as with other devices has not provided a perfect seal against moisture over long periods. For such purposes we have canned the jars, the vacant space around the top of the jar being filled with silica gel, to provide a double barrier with a dry space between.

It is sometimes an advantage to be able to dry simultaneously 5–10 ml. amounts of material in the 1 oz. screw-cap bottle in this machine, and for this purpose adaptors each holding four bottles can be placed in the carriers as required. The material is then, of course, dried in the wedge form.

Type 3 machine to hold one large container

This machine was designed for the freeze-drying of a large volume of solution in one operation. From Fig. 5 it is seen that the general layout, vacuum chamber, condensing coil, etc., are similar to that of the type 2 machine. The carrier, however, consists of a single large mild-steel cylinder welded to a stout base to accommodate a cylindrical container 18 in. diam. by 18 in. high. The base of the carrier is welded to a 2 in. shaft, the whole being turned true in the lathe and carefully balanced before assembly. Adequate bearings and robust construction are essential with the large liquid load (maximum of 15 l.) carried. The container is made of anodized spun aluminium, and Fig. 6 shows the container together with its perforated lid, to which a cotton-wool cover may be attached when sterile conditions must be maintained.

The drive is transmitted by twin V belts from a $\frac{1}{3}$ h.p. motor to a pulley on the 2 in. shaft, which is drilled throughout its length in order to convey the thermocouple leads from the carrier to the slip rings at the lower extremity of the shaft, a wax seal being arranged at this point. Heat is supplied by a concentric chromium-plated cylinder which carries nine vertical strip heaters symmetrically disposed, each strip having a maximum loading of 250 w. A horizontal metal reflector beneath the carrier restricts heat exchange with the condenser. To cut off the supply of heat as the product approaches dryness, a radiation thermostat (an adaptation of the usual domestic hot-water type) is fixed at the centre of rotation and set to switch off the heater load at a suitable temperature (usually about 20°). These features are seen in Fig. 7, which is a view from above with the lid swung aside. The thermostat, although somewhat crude, is simple and certain in action. The heat input is controlled by a Variac. The compressor, condenser and vacuum pump are similar to those used in the type 2 machine.

With a container of this height having vertical walls, the speed of rotation is determined by considerations of uniformity in the thickness of the liquid layer, the required centrifugal force being far in excess of that needed to suppress foaming. At the speed adopted, i.e. 500 rev./min. (centrifugal force of 65 *g* at the wall of the container) the difference in the thickness of the liquid layer between the top and the bottom of the vessel does not exceed $\frac{1}{4}$ in. Lower speeds could, of course, be used if the containers were made with the appropriate profile, tapering inwards towards the base.

With these large metal containers it is an advantage to maintain spinning throughout the freeze-drying process to ensure good thermal contact between the large mass of ice and the wall of the vessel. The more positive contact with the wall of the metal container thus achieved offers another marked advantage over the orthodox tray drier, where the mass tends to dry from beneath, as well as at the upper surface, thereby causing inclusion of ice within a cocoon of dried material and greatly hindering completion of the process. See, for example, R. Gane (see Harris, 1954, p. 189) and Bradish, Brain & McFarlane (1947).

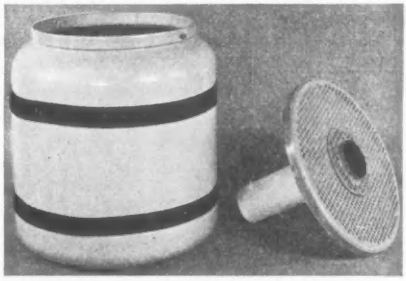

Fig. 6. Type 3 machine: container and lid.

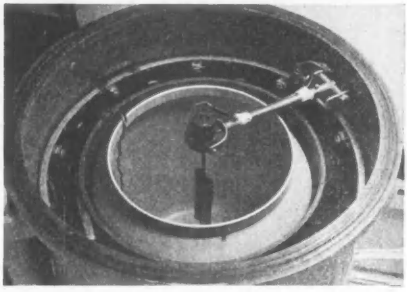

Fig. 7. Type 3 machine: view from above.

A container of the size used in this machine has the equivalent area of a tray drier of nearly 6 ft.², 13·5 l. producing a liquid layer with an average depth of 1 in. With a heat input of 1 kw and a test load of 13·5 l. of pure water, drying is complete in about 18 hr., which corresponds to an overall average drying rate of 0·75 l./hr., or 39 μg./cm.²/sec. Allowing for the rapid initial loss of water during the evaporative freeze, the steady rate during freeze-drying is about 32 μg./cm.²/sec. At this rate, with an open container, the temperature of the ice adjacent to the wall of the vessel is only about 1° higher than that of the condenser, and the drying rate can be substantially increased by raising the heat dissipated further to, say, 1·5 kw. This rate of drying compares very favourably with that quoted by Bradish *et al.* (1947) for a tray drier in which the condenser was adjacent to the drying material. It seems doubtful whether such close proximity of the condensing surface to the product does in practice result in any appreciable gain in drying rate, in view of the very small temperature differential between the subliming ice and the condenser surface in our equipment.

Adaptation for large-scale ampoule drying. The versatility of this type of machine is well illustrated by its application to ampoule drying. In place of the aluminium container, the carrier will accommodate several tiers of ampoule racks. Fig. 8 shows three tiers of racks, each rack holding 150 ampoules of the 1 oz. size. Each ampoule is inclined slightly to the centre so that its contents freeze in the wedge form. With 450 such ampoules, each containing 10 ml. of serum, i.e. a total load of 4·5 l., primary drying was completed in 48 hr. with a heat input of only 200 w. The speed of rotation (500 rev./min.) is more than sufficient to suppress foaming, even with ampoules arranged in the inner ring, although the drying of these ampoules in the middle of the centre rack is hindered somewhat by the restriction of their heat input by neighbouring ampoules. Racks cut from solid aluminium should offer some improvement in this respect.

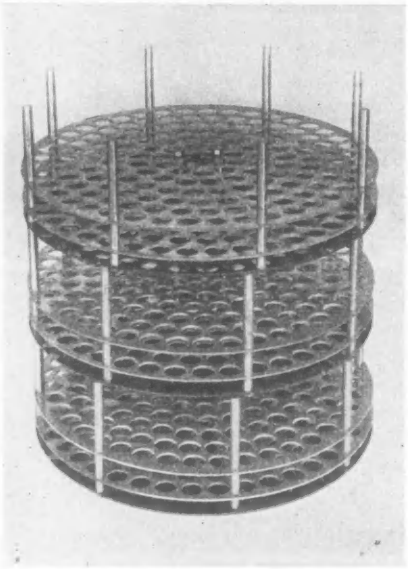

Fig. 8. Type 3 machine: ampoule racks.

Drying above the freezing point. In certain cases, where cell suspensions, etc., are required to be dried, freezing may not be tolerated without damage to certain enzymes, etc., and yet the suspension is required to be dried at the lowest possible temperature, e.g. just above 0°. The type 3 machine has been used for such a purpose by introducing an air leak, controlled by a needle valve, in the vacuum line to the pump. This maintains a constant pressure in the system, and at 5 mm. Hg, for example, and with continuous spinning to suppress foaming, 2 l. of a cell suspension was dried successfully overnight at +2° (±0·5°) without the application of any heat, to yield a compact, dry and friable product which still retained most of its original enzyme activity.

Special features of design

All three machines have certain features in common which have been arrived at by experiment and found, by actual use over a number of years, to be sound.

Lid seal. A very effective seal is achieved by a projecting lip on the underside edge of the lid, bearing upon a flat rubber ring cut from $\frac{1}{4}$ in. thick rubber sheet and located in a groove machined in the top rim of the chamber (Figs. 2, 5). When the chamber is evacuated the atmosphere exerts a pressure of about 3·7 tons on the lid, or 100 lb./in. length on the rubber ring.

Mainshaft seal. This is an adaptation of the orthodox type of seal employed on refrigerator-compressor crankshafts, and consists of a fixed portion (a spring-loaded metal bellows sweated to a phosphor-bronze ring) which seats upon the rotating portion (a case-hardened steel cup enclosing a Duprene rubber ring fitted snugly on the rotating shaft). This type of seal is effective only when running in oil and it is therefore enclosed in an oil reservoir which is connected by a pipe to an external sight glass, which may be topped up with oil occasionally as required. In order to ensure retention of oil in this reservoir, a second identical bellows seal is fitted back to back with the first one. A small air bleed, closed by a plug, at the top of the reservoir ensures complete filling with oil on assembly. Any vacuum leak at this shaft seal can occur only after the oil has first been drawn into the chamber, and this will be evident in the sight glass. The arrangement has given service over a period of many years without trouble, requiring topping up with oil at intervals of several months only.

Refrigerator-coil inlet and outlet seals. The inlet and outlet points of the low-temperature condenser from the vacuum chamber have often caused trouble in past designs owing to poor thermal insulation, and in some the expansion valve has been placed inside the chamber. This is undesirable for two reasons: (i) screwed refrigeration joints inside the vacuum chamber may allow leakage of gas which would affect the vacuum, contaminate the pump oil and be difficult to locate and repair; (ii) final adjustment of the regulator valve under working conditions is impossible. The difficulty has been overcome in the present designs by leading the two pipes into the chamber through Tufnol blocks of large dimensions, sealing the pipe by a rubber washer closed in on the pipe by a screwed collar. Although

the rubber washer probably becomes quite brittle at low temperature, no vacuum leaks have occurred at these points, and the seals have been undisturbed over a period of many years.

Vacuum inlet pipe. If the pumping of much water vapour and a poor vacuum are to be avoided this pipe must end in a region of low temperature in some position such as shown in Fig. 5.

Before opening the chamber after primary drying, air is admitted slowly through a needle valve. Freeze-dried materials, being in a fine state of subdivision, are usually very hygroscopic in nature and the admitted air is therefore dried by fitting the needle valve in the vacuum line to the pump so that the air entering the chamber first passes over the refrigerated coil, where most of its contained moisture is removed.

Measurement of temperature. A continuous record of the temperature of the material being dried (or of a representative sample) is important since the condenser temperature and the vacuum do not by themselves give sufficient indication of the temperature of drying and give negligible information about the rate of freezing, the final product temperature or the completion of drying. Multipoint recorders are commercially available which are robust yet sensitive enough to operate directly from copper–constantan thermocouples, and the simplicity, convenience and reliability of this method of temperature recording makes it the method of choice for freeze-drying purposes. Each machine has its own three-point thread recorder to give a continuous record of the temperature of the inlet and outlet of the condenser, and of the material being freeze-dried. The recorders are compensated for changes in ambient temperature, and no constant temperature ('cold') junction is required.

The temperature of the refrigeration-coil inlet and outlet is obtained by strapping the thermocouple, including several inches of the thermocouple leads, to the pipe at its point of entry into the chamber, the whole being well lagged. It is important that the thermocouples be insulated electrically from all metal parts of the equipment, as small stray earth currents can introduce serious errors.

In the present equipment, the measurement of product temperature presents a more difficult problem, since the current produced by the thermocouple e.m.f. must be conveyed from the rotating container via slip rings. The constantan line is picked up by the spring contact of a floating constantan brush bearing lightly against a central constantan rod turned to a point. In an earlier design the copper line was picked up by a copper brush bearing against a copper slip ring, and provided that the slip-ring diameter was kept small (0·25 in.) tolerable results were obtained with types 1 and 2 machines. In the type 3 machine, the thermocouple slip rings cannot conveniently be arranged inside the vacuum chamber, and it was found that at atmospheric pressure rapid oxidation and fouling of the copper slip ring rendered the device impracticable, especially as the container in this type is spinning throughout the drying operation. By the use of an auxiliary copper disk running freely in light ball bearings and in rolling contact with the copper slip ring, it is possible to make a point contact with the copper brush in the same way as employed for the constantan. This improved design (Figs. 9 and 4) is now giving excellent results on all three types of machine (Prov. pat. 12661/57).

At their point of exit from the vacuum chamber, thermocouple and heater leads are sealed by passing them through a cup containing vacuum wax (see, for example, Greaves, 1946). That part of each wire embedded in the wax must be stripped of its insulation over a short length and, where stranded conductors are used, the bared strands must be separated to avoid air channels in the wax. A layer of oil on the surface of the wax is effective in sealing minute cracks. Large temperature changes must be avoided by thermal insulation of the cup by the insertion of a short length of thick-wall rubber tube (see, for example, Fig. 5, type 3 machine). Although proprietary glass-to-metal seals are now available, they are unsuitable for thermocouple lines.

Measurement of vacuum. A continuous record of the pressure inside the freeze-drying chamber may be obtained by the use of the Pirani type of gauge, which is well suited to cover the pressure range concerned. Such a gauge must be calibrated against a standard, and even when calibrated the reading obtained depends to some extent on the ratio of air : water vapour in the system. With a continuous record of product temperature (or of a representative sample in types 1 and 2 machines), together with well-designed equipment in which vacuum troubles seldom occur, the continuous recording of vacuum is not essential. Reliable readings at intervals are nevertheless desirable and for this purpose an absolute gauge, the McLeod gauge,

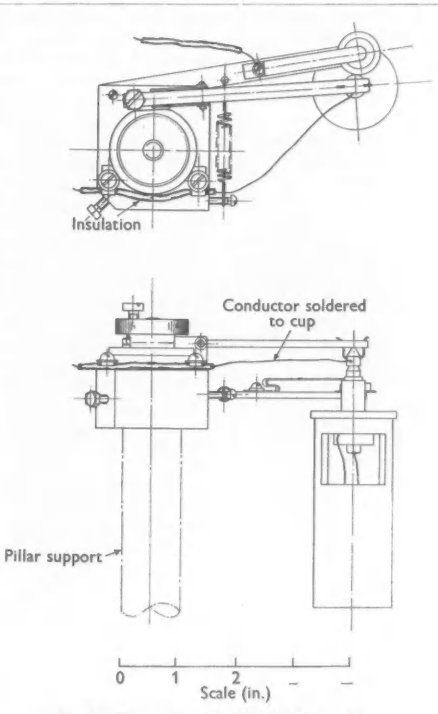

Fig. 9. Thermocouple slip-ring assembly.

has obvious advantages. Its use in an air–water-vapour system, however, is beset with pitfalls, and many controversial statements concerning its use on such systems are to be found in the literature. For example, Flosdorf (1949) states that provided that the measured pressure is below that of the 'condensation point' of the gauge, a true reading will be obtained. On the other hand, Greaves (1946) states that this type of gauge will neglect the water-vapour pressure altogether. In fact, the McLeod gauge reading obtained is somewhere between these two extremes, and it can be shown that the low readings obtained with this type of gauge on an air–water-vapour system are due mainly to adsorption of water vapour on the glass wall of the gauge during compression of the gas for measurement. Errors arising from deviations from the ideal gas laws in water vapour approaching saturation are of a relatively small order. The magnitude of the water-vapour adsorption error appears to depend on the condition of the glass; it is increased by cleaning the glass bulb and capillary with chromic acid, and a gauge head cleaned in this manner commonly gives readings of only 0·01 mm. Hg on an air-free system in which the ice-vapour pressure is known to be 0·055 mm. Hg (condensing coil temperature −45°). On the other hand, an identical gauge in which the bulb and capillary have been coated with molten paraffin wax *in vacuo* gives a true reading of 0·055 mm. Hg when connected to the same system. If reliable McLeod-gauge readings are to be obtained on an air–water-vapour system, such as that occurring in freeze-drying equipment, it therefore becomes imperative not only to use a gauge of such dimensions that no condensation of water vapour occurs when the gas is compressed for measurement, but further that adsorption of water on the glass of the gauge should be eliminated by treatment of the glass surface with a smooth water-repellent coating.

The occurrence of water-vapour adsorption errors appears to have been recognized by Ede (1947), who excluded moisture entirely from the gauge and measured only a balancing air pressure. Unfortunately, as the author points out, such a gauge will fail if the amount of air in the system falls below a certain level. By introducing a small quantity of glass wool into the upper part of the bulb of a McLeod gauge, we have been able to obtain, by a direct reading on the gauge used in a normal manner, a fair idea of the partial pressure of air in the freeze-drying chamber. Almost all the water vapour is adsorbed on the glass wool during compression of the gas for measurement, the gas being virtually dry by the time the mercury has risen into the capillary for measurement. The total pressure is determined on a separate identical gauge previously coated with paraffin wax as just described.

Both gauges were shown to give practically the same reading when connected to the same moisture-free system. On the other hand, typical readings when short lines were used for connexion to the vacuum chamber during freeze-drying were as follows: condenser temperature, −45°; total pressure (waxed gauge), 0·060 mm. Hg; air pressure only (glass-wool gauge), 0·002 mm. Hg. It is evident that practically all air has been swept out of the chamber during the drying process.

Defrosting procedure. For rapid defrosting of the refrigerated coil, provision is made for filling the lower half of the vacuum chamber from a hot-water supply. The removal of 15 l. of ice from the coil can be carried out in about 10 min. by this procedure.

Vacuum-pump exhaust. In the early stages of pumping down large chambers, the vacuum pumps, even when fitted with complex baffles or filters, throw up a fine aerosol of oil. Consequently, the exhaust from all pumps is piped outside the building.

PERFORMANCE

Evaporative spin–freeze. In evaporative freezing, in contrast with prefreezing where heat is abstracted via the wall of the containing vessel, cooling takes place at the free surface of the liquid and the ice layer starts to form at this interface, advancing outwards to the wall of the vessel at a rate depending on the surface temperature of the ice and limited by the thermal conductivity of the ice layer already formed. Thus for a given surface temperature, the rate of advance of the ice boundary will decrease progressively as freezing proceeds and complete and instantaneous 'snap' freezing can occur only with very thin liquid layers. With a condensing-coil temperature of −30°, and a vacuum of 0·29 mm. Hg equal to the vapour pressure of ice at this temperature, a 1 cm. thick ice layer is advancing at the rate of 1 mm./min. (a value of 0·005 cal./sec./cm.^{2}/° C for the thermal conductivity of the ice already formed being accepted). The freeze-drying of thick-liquid layers may therefore be undesirable with substances sensitive to concentrated salt solutions. It is, however, important to avoid the other extreme of too thin a liquid layer if drying from the frozen state is to be assured. It has already been shown that about one-sixth of the water in the product to be dried must be evaporated in order to cool the remainder from room temperature to −25°. This estimate makes no allowance for the heat capacity of the container and if this is large in relation to the volume of material to be dried, it may even be possible for all, or most, of the water to evaporate before freezing takes place. It is then desirable to increase the volume of the material by dilution. Some solutions, especially those containing material at high concentration or of high viscosity, hinder evaporative freezing owing to the formation of a surface skin of dried material; evaporation then practically ceases at the outset and the solution does not freeze. The remedy lies in diluting the solutions, where this is feasible, or in prefreezing where it is not.

Fracture of the containing vessel. One of the practical difficulties arising from the direction of ice formation in evaporative freezing is the tendency to cause cracking of the glass containing vessels, as a result of the expansion on freezing of the liquid trapped by the inner cylinder of ice first formed. Fracture occurs only with a certain minimal thickness of liquid layer in relation to the

dimensions and mechanical strength of the container, e.g. 200 ml./jar appears to be a safe volume for 3 lb. bottling jars under all conditions. With volumes in excess of 200 ml., fracture is most prone to occur with dilute solutions or suspensions in the absence of salts. Under the normal conditions of operation of the type 2 equipment, the presence of sodium chloride in solution affords complete protection with 400 ml./jar even at concentrations as low as 0·025 %, evidently by weakening the ice structure by the presence of a liquid layer between the ice crystals, and it is usual to find evidence of slight bulging of the ice surface at one or two points when freezing has been completed. The protection afforded by various salts appears to depend on their eutectic points in relation to the conditions of freezing. For example, in tests carried out in the type 2 machine under normal operating conditions, potassium chloride, with a eutectic point at −11·1°, was far less effective than sodium chloride (eutectic point at −21·2°) in preventing fracture. At a slower rate of freezing, on the other hand (e.g. by the introduction of an air bleed into the system), potassium chloride may prove equally effective.

The tendency of ampoules to fracture when material is dried in the wedge form depends on the tenacity with which the ice layer can key itself to the glass wall. For this reason solutions of gelatin are very prone to cause trouble, owing to the strong adherence of gelatin film to a glass surface. Such a film would be formed at the edges of the wedge of material in the early stages of evaporative freezing, and would serve to key the ice layer firmly to the ampoule. It was found that the trouble could be eliminated by pretreatment of the ampoule interior with one of the organosilanes. This particular hazard with gelatin concerns only the wedge form of evaporative freezing and does not arise when the container is spun about its own axis.

'*Spill.*' 'Spill' (Cowan, 1951), or ejection of the dried material from the container as drying proceeds, is another hazard which depends upon the nature of the material being dried. Since 1 g. of water vapour at a pressure of 0·1 mm. Hg has a volume of about 10^4 l., the rate of evolution of water vapour from a single 3 lb. bottling jar in the type 2 machine amounts to over 50 l./sec., and this may cause portions of the dried outer layer to be ejected with almost explosive violence. In the freeze-drying of a 1 % sodium chloride solution about half of the salt is ejected in this manner, whereas serum appears to suffer no loss. The usual sterile-filter caps reduce the effect by decreasing the vapour flow as a result of the raised water-vapour pressure inside the bottle. Such filters also trap most of the material that is ejected, but are not completely effective. By drying under the influence of centrifugal force, as in the normal operation of the type 3 machine (force 65 *g*), 'spill' is almost completely suppressed, a 1 % sodium chloride solution drying under these circumstances with negligible loss.

Size, shape and distribution of the ice crystals. When moderate concentrations of proteins, etc., are properly freeze-dried, i.e. from the completely solid state, the resulting dried product retains not only the volume of the original solution but also the detail of the ice-crystal pattern laid down during the freezing stage. Microtome sections of the dried material, after embedding in paraffin wax under vacuum, reveal a network lattice of dry protein delineating the boundaries of the original ice crystals (Fig. 10). Except for a random distribution in the surface layers, the bulk of the ice is laid down as a mass of thin laminae perpendicular to the wall of the container and thus parallel to the temperature gradient during freezing. In this respect the pattern closely resembles that observed in freezing by ordinary methods of cooling (see, for example, Dorsey, 1940, p. 411). Under standard conditions of freezing in the type 3 machine, a 10 l. load of 5 % ovalbumin showed that the resulting ice-crystal laminae had a fairly uniform thickness of about 0·006 mm.

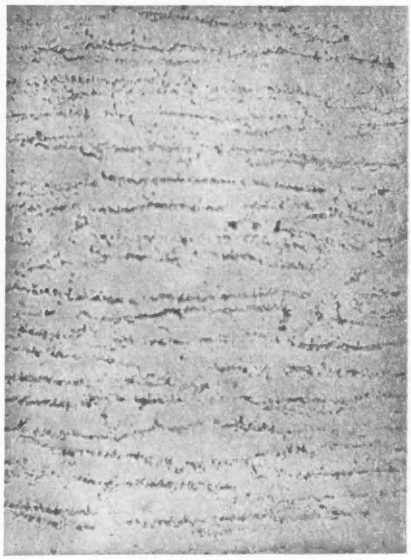

Fig. 10. Microtome section of freeze-dried 5 % ovalbumin.

A typical drying cycle. Fig. 11 shows a routine temperature record obtained during the freeze-drying of 10 l. of 5% ovalbumin in the type 3 machine. The temperatures of the inlet and outlet of the condensing coil are almost coincident and are shown by the lower trace. The product temperature is recorded on the upper trace. Evacuation of the chamber began at time 0, with the coil temperature at −45° and the product at room temperature (20°). Reduction of the chamber pressure to a few mm. Hg was marked by a rapid fall in product temperature to 0°, near which it remained for the next 20–25 min., until the freezing of almost all the water was complete. The temperature of the mass of ice then dropped sharply until it was superimposed on that of the condenser. Measurements with a thermocouple which, together with about 12 in. of the thermocouple leads, was embedded in a layer of ice frozen on to the condensing coil inside the chamber, showed that the actual condenser temperature was in fact approximately 2 degrees lower than that indicated by the coil temperature on the chart. The product temperature in the absence of any applied heat other than from stray radiation and conduction derived from the surroundings was therefore about 2 degrees higher than that of the condenser.

After switching on the heaters (1 kw loading) at 2 hr., steady-state conditions were attained, with a product temperature adjacent to the wall of the container some 5–6 degrees higher than that of the condenser inlet, i.e. 7–8 degrees higher than the true condenser temperature. As drying proceeds the temperature of the product gradually rises as a result of the impedance to vapour flow presented by the increasing thickness of the network of dried material. The magnitude of the impedance is dependent not only on the thickness of the dried layer above the ice surface but also on the size of the ice crystals or laminae laid down in the freezing stage, which govern the size of the channels in the dried mass through which the water vapour makes its escape. The final stages of drying are completed relatively quickly, there being no evidence of the prolonged second phase described by Bradish *et al.* (1947) in the orthodox tray drier. The product, when removed from the machine at the end of the recording, showed complete absence of wet spots.

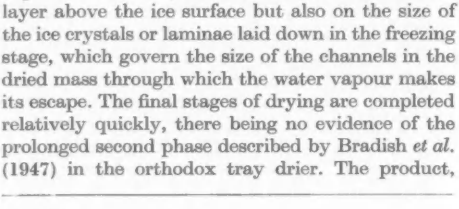

Fig. 11. Temperature record of freeze-drying of 10 l. load in type 3 machine.

An indication of the amount of obstruction to vapour flow in the absence of a layer of dried material may be obtained by drying a 10 l. load of pure water under the same conditions of vacuum, applied heat (1 kw), etc. The subliming ice then assumes a constant temperature within only 1° of the actual condenser temperature; this suggests that the gain in rate of drying which would be obtained with a condenser in close proximity to the product would be negligible. Whereas the drying temperature for water is markedly lower than that for 5% ovalbumin solution, the total drying time is approximately the same. These results depend, of course, on the maintenance of a vacuum equal to that of the vapour pressure of ice at the temperature of the condensing coil; any rise of pressure in the chamber would at once lead to a rise in the product temperature.

SUMMARY

1. Equipment is described for the routine freeze-drying of material on a large laboratory scale with the minimum of handling and attention. The evaporative spin–freeze principle is used throughout.

2. Details are given of various special features of design, and of the methods used for the measurement of the temperature of drying material in rotating containers, and of the vacuum in low-pressure air–water-vapour systems.

3. The practical advantages and disadvantages of this type of equipment are discussed and the performance is illustrated by a typical freeze-drying cycle.

Our thanks are due to Mr S. H. Fryer for the use of the facilities of the Engineering Section in the construction of the equipment herein described, and to Mr S. H. Offer for much valuable help in its design and execution. We are indebted to Dr D. W. Henderson for his interest and encouragement in this work.

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Oxidative Phosphorylation by Subcellular Particles from Yeast

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The presence in *Saccharomyes cerevisiae* of refractile cytoplasmic granules with staining properties resembling those of mitochondria from animal cells has been reported by Mudd *et al.* (1951) and Hartman & Liu (1954). On the basis of earlier evidence Lindegren (1949) had suggested that the cytoplasmic granules of yeast were mitochondrial in nature. This hypothesis has been supported by the findings of Slonimski & Ephrussi (1949) that subcellular particles containing the enzymes concerned with terminal oxidation can be obtained by differential centrifuging of broken yeast cells. As further evidence Nossal (1954) obtained subcellular particles which contained several dehydrogenases and accessory enzymes of the Krebs cycle, and Linnane & Still (1955) obtained a similar fraction able to oxidize pyruvate.

In the present report, the ability of subcellular particles from yeast to couple oxidation with phosphorylation has been demonstrated. This finding adds strong evidence in support of the view that the particles are related biochemically to mitochondria, since the ability to carry out respiratory-chain phosphorylation is perhaps the most definitive biochemical characteristic of mitochondria.

Oxidative phosphorylation by cell-free preparations from micro-organisms has been demonstrated previously by Pinchot & Racker (1951), using *Escherichia coli*, by Pinchot (1953), using *Alcaligenes faecalis*, by Tissières & Slater (1955), using *Azotobacter vinelandii*, by Brodie & Gray (1956*a*, *b*), using *Mycobacterium phlei*, and by Nossal, Keech & Morton (1956*a*), using *Proteus vulgaris*. In all these studies a soluble component has been implicated in the system employed, in contrast to the present studies with yeast, where washed particles were able to couple phosphorylation to oxidation without supplementation. In addition, the phosphorylation of the yeast particles was uncoupled by 2:4-dinitrophenol, whereas all of the bacterial systems with the exception of *Mycobacterium phlei* (Brodie & Gray, 1956*a*) are resistant to the action of this uncoupling agent.

METHODS

Preparation of particles. The bakers' yeast used in these experiments was obtained from Effront Ltd. (South Yarra, Victoria, Australia) or Red Star Yeast and Products Co. (Milwaukee, U.S.A.) as the pressed, cooled cake and stored until use in a humid atmosphere at 2–5°. The yield and activity of the granules seems to depend somewhat on the age of the yeast, but generally satisfactory results were obtained any time from 3 to 10 days after harvesting.

The yeast was suspended evenly in cold 1% NaCl soln. containing 0·01–0·001 M-ethylenediaminetetra-acetic acid (EDTA), pH 6·5, in a ratio of 1·5 g. of yeast cake/7 ml. of suspending fluid. The suspension (10–14 ml.) was added to pre-cooled glass beads (Ballotini no. 12; C. A. Brinkmann and Co., Great Neck, Long Island, New York) in a steel capsule, with 2 ml. of yeast suspension/g. of glass beads. The cells were disintegrated by shaking the capsules for 5–10 sec. at 0° in a high-speed mechanical disintegrator (Nossal, 1953), at 6000 cyc./min. Such treatment ordinarily disrupts 20–40% of the yeast cells. If several capsules are available for use with the disintegrator about 90 g. of yeast can be treated in 20 min. by two operators.

The suspension of broken and whole cells was decanted from the glass beads and the suspension adjusted to pH 6·8 by the cautious addition of dil. NaOH soln. or 0·4 M-2-amino-2-hydroxymethylpropane-2:3-diol. Most whole cells and debris were removed by centrifuging for 10 min. at 0° and 1000 *g*. This process was repeated to remove the remaining whole cells, or the same end was achieved by using only the top two-thirds of the supernatant resulting from the first centrifuging. The subcellular particles used in these studies were obtained by centrifuging the supernatant at 13 000–23 000 *g* for 15 min. at 0°, in a refrigerated angle centrifuge. Since centrifuging at 10 000 *g* is sufficient to recover most of the particles, variations above this value are unimportant. The pellet was resuspended in either 1% NaCl or 10% sucrose, each containing 0·001 M-EDTA, and the high-speed centrifuging step repeated. Ordinarily, the particles were washed only once, but the washing process can be repeated several times without significant change in the properties which have been studied. The washed pellet was carefully suspended in one-third to one-sixth of the original volume of 1% NaCl or 10% sucrose, each containing 0·001 M-EDTA and 0·01 M-sodium or -potassium phosphate (pH 6·7). As is discussed below, the properties of the particles are influenced somewhat by the nature of the washing and suspending media.

Manometric methods. About half of the experiments were conducted in Warburg flasks of approx. 20 ml. volume with a reaction mixture containing the following components in a total volume of 2·25 ml.: 50 μmoles of sodium succinate, 2 μmoles of adenosine triphosphate (ATP), 25 μmoles of glucose, 20 μmoles of NaF, 10 μmoles of $MgCl_2$, 10 μmoles of inorganic phosphate (P_i) (added with particles), 1 μmole of EDTA (added with particles), 0·3 unit of hexokinase, 50 μmoles of glycylglycine or tris buffer (pH 6·7) and 1 ml. of yeast particles with a protein content of 5–8 mg. The remaining experiments were

carried out in vessels with a total volume of approx. 10 ml., 1·3–1·6 ml. being used as the total fluid volume and with the various components scaled down accordingly. In the experiments with particles from Red Star yeast, 2–4 mg. of bovine albumin was added since this substance gave increased P/O values with most preparations. The source of yeast, disintegration time, washing and suspending media and deviations from the assay procedure just described are noted for individual experiments. Experiments were conducted at 22–23°, with air as the gas phase, with experimental flasks in duplicate and a single zero-time control for each pair.

All components of the assay mixture were added to the main chamber of the vessel and pre-cooled in ice before addition of the hexokinase and yeast particles. The centre wells of the vessels contained 20% KOH soln. and fluted filter paper, and one side bulb contained 0·1 ml. of 10N-H_2SO_4 or 0·2 ml. of 10% trichloroacetic acid. After addition of the particles the vessels were attached to the manometers as quickly as possible and equilibrated for 10 min. at 22–23°. At the end of this period, the reaction was terminated in the control vessels by tipping in the acid from the side bulb and manometric measurements were initiated with the experimental vessels.

Determinations. Glucose 6-phosphate was determined spectrophotometrically by the use of the specific dehydrogenase on trichloroacetic acid filtrates neutralized with NaOH soln. Determinations were carried out at 30° in 3 ml. of assay mixture containing 100 μmoles of glycylglycine (pH 7·4), 3 μmoles of $MnCl_2$, 0·6 μmole of triphosphopyridine nucleotide (TPN), 0·2 unit of glucose 6-phosphate dehydrogenase and 0·03–0·15 μmole of glucose 6-phosphate. In our hands some preparations of the dehydrogenase were not satisfactory for this assay and consistently yielded low results, and it was necessary to test preparations carefully on known solutions before use. The presence of trichloroacetate slows the rate of production of reduced TPN somewhat and was not permitted to exceed 0·01 M. In general, best results were obtained from conditions arranged to permit completion of the reaction in 15 min. or less.

Fumarate and malate were determined spectrophotometrically on trichloroacetic acid filtrates neutralized with NaOH soln., by using malic enzyme and fumarase and measuring production of reduced TPN. The assay mixture contained the following components in a volume of 3 ml.: 150 μmoles of glycylglycine (pH 7·4), 2 μmoles of $MnCl_2$, 0·6 μmole of TPN, 500 μmoles of semicarbazide (pH 7·4), 0·4 unit of malic enzyme and 2 units of fumarase. The semicarbazide is required to prevent oxidation of reduced TPN by pyruvate and lactic dehydrogenase, since the latter is present as a contaminant of malic enzyme. At 30° 0·05–0·2 μmole of fumarate and malate can be estimated in a reaction period of about 30 min. The preparations of malic enzyme employed contained as much lactic dehydrogenase [measured with reduced diphosphopyridine nucleotide (DPN) and pyruvate] as malic enzyme, and although reaction with reduced TPN is much slower and the pyruvate is bound by the semicarbazide, the values for malate and fumarate obtained were slightly less than predicted.

Orthophosphate (P_i) was determined on trichloroacetic acid filtrates by the method of Lohmann & Jendrassik (1926) and protein was determined by the colorimetric biuret method of Robinson & Hogden (1940), with crystalline bovine albumin (Armour) as a reference standard.

Enzymes. Hexokinase was prepared from yeast by following the procedure of Berger, Slein, Colowick & Cori (1946) as far as step V, and stored in glucose and phosphate buffer (pH 5·4) at −19°. As used in this paper, 1 unit of activity corresponds to the utilization of 1 μmole of substrate or formation of 1 μmole of product/min. Before use the hexokinase was diluted with 9 vol. of 1% cold albumin and 0·1 ml. of the diluted solution was used in each reaction vessel. Malic enzyme was prepared from chicken liver as described by Utter & Kurahashi (1954) and had a specific activity of 0·3 unit/mg. of protein. Fumarase was prepared from pig heart by the procedure of Massey (1955) as far as the first ammonium sulphate step after the gel treatment, and had a specific activity of 137 units/mg. of protein as measured by the spectrophotometric assay of Racker (1950). Glucose 6-phosphate dehydrogenase was prepared from yeast as described by Kornberg (1950) and had a specific activity of 1·5 units/mg. of protein.

Other materials. ATP, adenosine diphosphate (ADP), DPN and TPN were commercial materials obtained from Pabst Brewing Co., and cytidine diphosphate (CDP), guanosine diphosphate (GDP), uridine diphosphate (UDP), and inosine triphosphate (ITP) were commercial materials obtained from Sigma Chemical Co. 2-Deoxyglucose was synthesized by the method of Cramer (1955) and was generously provided by Mr P. Russell.

RESULTS

Components of the system. Particles obtained by differential centrifuging of disrupted yeast cells are able to couple phosphorylation with the oxidation of succinate (Table 1). Phosphorylation is greatly depressed or abolished by the omission of ATP, hexokinase or $MgCl_2$, although oxidation is unaffected or even increased by the omission of these substances. The washed particles exhibit little phosphorylation or oxidation in the absence of added substrate. The presence of NaF had little effect upon the reaction in this experiment, but NaF was always included since early experiments had demonstrated the presence of adenosine triphosphatase in the particles.

Table 1. *Oxidative phosphorylation with yeast particles*

Particles were prepared from Effront Yeast by 10 sec. disintegration and washed and suspended in NaCl–EDTA. Approx. 8 mg. of protein was used in a total volume of 2·25 ml. with the assay procedure described in the text. The incubation time (not including equilibration period) was 30 min.

Components	O_2 uptake (μg. atoms)	P_i uptake (μmoles)	P/O
All	6·35	3·93	0·62
ATP omitted	6·8	1·01	0·15
$MgCl_2$ omitted	8·2	1·2	0·15
Hexokinase omitted	7·3	−0·88	0
NaF omitted	6·8	4·1	0·61
Succinate omitted	0·8	0·6	0·8

Table 2. *Uncoupling of phosphorylation by 2:4-dinitrophenol*

Particles were prepared as described in Table 1 and assayed as described in the text, with an incubation period (not including equilibration period) of 20 min.

2:4-Dinitrophenol (mM)	O_2 uptake (μg. atoms)	P_i uptake (μmoles)	P/O
0	3·4	2·25	0·66
0·036	3·45	1·6	0·46
0·091	3·4	0·85	0·25
0·18	3·3	0·2	0·06
0·36	3·15	−0·15	0

Over a large number of experiments, the P/O ratios observed for the oxidation of succinate varied from 0·4 to 1·2, with the majority of values falling between 0·6 and 0·9. In occasional experiments there was failure to observe phosphorylation or even oxidation. The negative experiments were traced mainly to improper disintegration or pH control procedures. The variables controlling the yield and activity of the yeast particles and especially the P/O ratios are only partially understood, but include; disintegration time; pH during isolation of the particles and during the assay procedure; the presence or absence of certain substances such as albumin, sucrose and P_i during handling procedures; the maintenance of an optimum level of ADP during assay; and the age and condition of the yeast. The first two of these factors are dealt with in subsequent experiments in this paper and the third and fourth factors are under investigation at present. The P/O values are considerably lower than reported for succinate oxidation with animal mitochondria (see, for example, Maley & Plaut, 1953) and from this discrepancy, together with the observation (Table 1) that there is no obligatory coupling between oxidation and phosphorylation with the yeast particles, it might be inferred that the particles are not in their native state.

Uncoupling by 2:4-dinitrophenol. Although phosphorylation connected with respiration in several bacterial cell-free systems has been reported to be insensitive to 2:4-dinitrophenol, the yeast system is completely uncoupled by this substance (Table 2). The concentration required for complete uncoupling, 0·3–0·5 mM, is five- to ten-fold higher than that reported for animal mitochondria by Cross, Taggart, Covo & Green (1949). In agreement with the finding that the presence of an ATP-trapping system does not stimulate oxidation (Table 1), the addition of 2:4-dinitrophenol fails to enhance the oxidation of succinate, in fact, at higher concentrations, oxidation of succinate is slightly inhibited.

The effect of 2:4-dinitrophenol can be prevented by its removal from the particles by washing (Table 3). In these experiments after the whole cells and debris had been removed by preliminary centrifuging, 0·6 mM-2:4-dinitrophenol was added to one half of the supernatant but the other half was untreated. Both portions were then centrifuged to obtain particles, which were washed with NaCl–EDTA solution and resuspended in the same medium. When tested with succinate, the particles obtained from the 2:4-dinitrophenol-treated supernatant had lost some respiratory activity, but the P/O value was slightly higher than that of the control particles. The oxidative phosphorylation of both types of granules was uncoupled by 2:4-dinitrophenol added during the assay.

Variations in the preparation of the particles. The duration of the disintegration period has a marked effect on the particles subsequently obtained (Table 4). The second and third columns of Table 4 show the protein content of the initial supernatant fluids and the amount of protein obtained by centrifuging respectively. The fourth column shows the oxidative activity exhibited by similar quantities of particles obtained with the various shaking periods. There is some variation in oxidative activity, but the most notable effect is a constant decrease in the P/O ratio as the period of shaking is increased. The loss of phosphorylative activity may be due to further disruption of the particles, since Nossal (1954) has found that with longer periods of shaking several enzymes, including fumarase, aconitase and ethanol dehydrogenase, tend to move from the particulate fraction to the supernatant fraction.

The composition of the medium in which the yeast cells are disrupted and in which the particles are washed and suspended has a considerable influence upon the properties of the particles. In these experiments 1 % NaCl soln. containing EDTA has been generally employed as the medium for disintegration. Particles obtained by disintegration in alkaline phosphate have been found by Nossal *et al.* (1956*a*) to be able to oxidize a number of substrates including succinate. However, phosphate has been shown to have a deleterious effect on mitochondrial integrity (Hunter & Ford, 1955) and the phosphorylation of yeast particles is also adversely affected by the presence of phosphate, so this disintegration procedure is unsuitable for the present studies. Preliminary studies with 0·9 % KCl soln. yielded particles with considerable endogenous activity and with lower P/O values than similar preparations obtained with NaCl. Linnane & Still (1955) used sucrose for the disintegration of yeast cells and obtained particles capable of carrying out the complete oxidation of pyruvate. Sucrose has not been investigated extensively in the present studies, but the results available indicate that particles obtained from disintegration in

Table 3. *Reversibility of inhibition by 2:4-dinitrophenol*

Particles were prepared from Effront Yeast by 10 sec. disintegration. To one half of the supernatant 2:4-dinitrophenol was added to give a final concentration of 0·6 mM and the other half was untreated. Particles were recovered from both portions by centrifuging at 20 000 *g*, washed with NaCl–EDTA, resuspended in NaCl–EDTA and assayed for 30 min. as described in the text.

Treatment of particle	2:4-Dinitrophenol (mM)	O_2 uptake (μg. atoms)	P_i uptake (μmoles)	P/O
None	0	7·3	3·2	0·44
None	0·54	6·2	0	0
2:4-Dinitrophenol	0	4·4	2·4	0·55
2:4-Dinitrophenol	0·54	4·6	0·4	0·07

Table 4. *Effect of disintegration time*

Particles were prepared from Red Star Yeast and were washed and suspended in sucrose–EDTA. Particles equivalent to 2·5 mg. of protein were assayed as described in the text, for 30 min. in a total volume of 1·3 ml.

Disintegration time (sec.)	Protein* (mg.) Initial supernatant	Particles	O_2 uptake (μg. atoms)	P_i uptake (μmoles)	P/O
5	6·4	1·4	2·34	1·29	0·55
10	10·2	2·7	1·58	0·73	0·46
20†	15·1	5·0	1·74	0·54	0·31
40†	20·7	5·7	1·93	0·40	0·21

* Yield/ml. of initial supernatant from centrifuging at 1000 *g*. † With cooling after each 10 sec. interval.

sucrose exhibit P/O values of the same general range as those obtained by disruption in NaCl. The presence of EDTA aids greatly in the stabilization of yeast particles, as has been reported for animal mitochondria (Slater & Cleland, 1952).

Two different washing and suspending media have been employed in these studies: 1 % NaCl and 10 % sucrose, each containing 0·001 M-EDTA. Although the particles obtained by these alternative procedures have generally similar characteristics, particles prepared in sucrose seem to have a higher activity/mg. of protein, although P/O values are no higher than with granules prepared in NaCl. The particles prepared in sucrose appear to be associated to some degree with several pyridine nucleotide dehydrogenases, whereas the particles prepared in NaCl are virtually free from the same dehydrogenases (see below). The presence of sucrose or albumin or both during the washing and suspending procedures seems to stabilize the particles, and the influence of these and other substances is under further investigation.

Sedimentation behaviour of yeast particles. In the foregoing experiments the particulate fractions were obtained from the centrifugal interval between 1000 *g* and 13 000–23 000 *g*. The fractions used possibly contained sedimentable material of greatly varying size and activity. To determine whether the oxidative and phosphorylative activities were correlated in any regular fashion with sedimentation properties, fractionation studies were attempted. Sedimentable material was collected by centrifuging for successive 15 min. periods at 5000, 10 000 and 23 000 *g*. Each centrifuging was carried out in 0·25 M-sucrose in an angle-head centrifuge, and the relative centrifugal forces as given apply to the bottom of the centrifuge tubes. After washing, each fraction was tested on succinate along with another portion which was obtained by centrifuging directly at 23 000 *g* (Table 5). Fraction *A*, representing material thrown down over the entire range from 1000 to 23 000 *g*, gives essentially the same results as the material sedimented in a similar time interval at 5000 *g* (fraction *B*). The material sedimented between 5000 and 10 000 *g* (fraction *C*) has a slightly lower oxidative activity and a slightly lower P/O value. Fraction *D*, representing the material sedimentable with difficulty shows a markedly lower oxidative activity, although the P/O value is still comparable. From the second column of Table 5, in which the yield of particles is given, it can be seen that about 55 % of the sedimentable protein is obtained in fraction *B*, about 25 % in fraction *C* and somewhat less in fraction *D*. According to these preliminary studies, there is no indication of a significant fractionation of the material responsible for oxidative phosphorylation.

The experiments shown in Table 5, and several other attempted fractionations, indicate that the active material is distributed over a considerable range of centrifugal behaviour, and that half or more of the sedimentable protein can be obtained in 15 min. by centrifugal forces not exceeding

Table 5. *Centrifugal fractionation of particles*

Particles were obtained from Red Star Yeast by 5 sec. disintegration and the supernatant from the initial centrifuging at 1000 *g* was fractionated as described in the text after the addition of sucrose to a concentration of 0·25 M. Each fraction was washed and suspended in sucrose–EDTA and incubated with succinate for 30 min. in a total volume of 1·3 ml. Fractions *A* and *B* were assayed with 2·2 mg. of protein/vessel and *C* and *D* with 2·9 mg. of protein/vessel. Here and in Table 6 the incubation time does not include the equilibration period.

Fraction	10^{-3} × Relative centrifugal force (*g*)	Yield* (mg.)	O_2 uptake (μg. atoms)	P_i uptake (μmoles)	P/O
A	1–23	0·72	3·05	2·26	0·74
B	1–5	0·45	3·15	2·31	0·73
C	5–10	0·2	3·14	1·98	0·73
D	10–23	0·14	1·47	0·93	0·63

* Protein in particles/ml. of supernatant from centrifuging at 1000 *g*.

5000 *g*. The 'respiratory granules' of yeast, as identified by microscopic observation (Mudd *et al.* 1951), have been reported to range from 0·3 to 1 μ in diameter. Linnane & Still (1955) estimated that the particles obtained by disintegration in sucrose were about 0·3 μ in diameter, although the fraction used was precipitated between 3000 and 25 000 *g* and no fractionation studies were reported. These observations can be reconciled with the present studies by assuming that the active particles are small in size but are associated to varying degrees with larger structural elements of the yeast cell under the disintegration conditions used. It should be noted that the period of shaking was only 5 sec. in these experiments and that it is possible that longer periods of shaking would change the centrifugal distribution. A second possible explanation for the present results is that the active particles are small but tend to aggregate under the conditions used for isolation. Preliminary studies of some of the fractions by electron and phase-contrast microscopy lends some support to the hypothesis that small particles are attached to a larger structural element such as a vacuolar membrane.

Whole yeast cells can be essentially eliminated from consideration in these studies, since parallel experiments with yeast suspensions treated in the usual way except for omission of disintegration show that at most 1–2 % of the sedimented protein is in the form of unbroken cells. Direct examination with the phase-contrast microscope also indicates that the whole cells are very few in number in the fractions.

Effect of pH. Since oxidation and phosphorylation are not obligatorily associated in the yeast particles, it is possible to investigate the two processes as separate phenomena. The effect of variations in pH during the assay is shown in Table 6. The results show that the oxygen uptake increases markedly as the pH is increased from 6·3 to 7·5, whereas phosphorylation shows a broad maximum between 6·6 and 7·5. On the basis of

Table 6. *Effect of* pH

Particles were prepared from Effront Yeast by 10 sec. disintegration and the particles isolated without the usual neutralization. The particles were divided into portions and washed in NaCl–EDTA containing 0·016 M-sodium phosphate of the appropriate pH and suspended in the same medium. Particles equivalent to 5 mg. of protein were incubated with succinate for 20 min. with components as described in the text except that glycylglycine and succinate were adjusted to the pH indicated.

Expt. no.	pH	O_2 uptake (μg. atoms)	P_i uptake (μmoles)	P/O
1	6·3	2·9	1·8	0·62
	6·6	4·35	3·0	0·69
	6·9	5·0	3·0	0·60
2	6·6	3·6	2·8	0·78
	7·2	6·65	3·5	0·52
	7·5	7·45	3·1	0·41

P/O ratios, the pH range of 6·6–6·9 is the most satisfactory and has been used in these studies.

Specificity of phosphate acceptor. The yeast particles seem to have an absolute specificity for ADP as the phosphate acceptor (Table 7). Only ADP or ATP (which in the presence of hexokinase and glucose is converted into ADP) can cause phosphorylation, and CDP, UDP, GDP and ITP are all completely inactive. The presence of the other nucleoside diphosphates has little or no inhibition of phosphorylation in the presence of ADP and the oxygen uptake is not affected. The specificity for ADP is in agreement with the results of Cooper & Lehninger (1956), who found that ADP is the obligatory acceptor for phosphorylation with mitochondrial fragments of rat liver. Herbert, Potter & Takagi (1955) have reported that mitochondria from rat liver can phosphorylate UDP to UTP during oxidation, but it is by no means certain that UDP is the primary acceptor.

In Expt. 3, of Table 7, it is shown that adenosine monophosphate (AMP) cannot replace ADP as the acceptor in the yeast system. Preliminary studies indicate that at concentrations of ADP lower than

used in Table 7, AMP inhibits phosphorylation. This finding is apparently in distinct contrast with studies with animal mitochondria (Lehninger, 1953), where AMP can serve successfully as the phosphate acceptor, presumably through the mediation of myokinase, in the absence of NaF.

Products formed during oxidation of succinate. Under the conditions of the present studies the oxidation of succinate is essentially a one-step reaction to fumarate. In the experiments of Table 8, the accumulation of fumarate and malate is compared with the consumption of oxygen and P_i. Since it had been found in experiments not shown here that particles prepared by washing with sucrose contained appreciable quantities of glucose 6-phosphate dehydrogenase, parallel experiments were included here in which 2-deoxyglucose was substituted for glucose in the ATP-trapping system. Woodward & Hudson (1955) have shown that yeast hexokinase phosphorylates 2-deoxyglucose and glucose at comparable rates. However, the further metabolism of the phosphorylated product should be much slower with 2-deoxy sugar, and thus the latter is the preferred acceptor in many situations.

Table 7. *Specificity of phosphate acceptor*

Particles were prepared and tested as described in Table 1, except that 2 μmoles of the various nucleoside polyphosphates were added as indicated.

Expt. no.	Acceptor	O_2 uptake (μg. atoms)	P_i uptake (μmoles)	P/O
1	ATP	6·8	3·0	0·44
	ADP	5·5	2·9	0·53
	UDP	6·4	0	0
	UDP + ADP	7·5	2·6	0·35
	CDP	6·9	0	0
	CDP + ADP	7·6	3·2	0·42
2	ADP	7·1	3·4	0·48
	ITP	6·4	0	0
	ITP + ADP	8·3	3·4	0·41
	GDP	6·4	0	0
	GDP + ADP	8·5	3·9	0·46
3	ADP	6·0	4·1	0·68
	AMP	4·8	0	0
	AMP + ADP	5·5	4·5	0·82

From the first two lines of Table 8, it is seen that there is little consumption of oxygen or P_i in the absence of succinate. After a 30 min. reaction period, a considerable portion of the glucose 6-phosphate which had been formed during the equilibration period by hexokinase action had disappeared. From this amount, 0·93 μmole, it might be expected that the oxygen uptake in line 1 should be at least 0·45 μmole instead of 0·2 μg. atom, but these values are so small as to be within experimental error. With glucose as the acceptor about 70 % of the theoretical amount of fumarate and malate predicted from oxygen uptake is found. With 2-deoxyglucose the amount of fumarate and malate rose to 85 % of theoretical. As discussed under Methods, the determination employed gives slightly low values for malate and fumarate, and it seems safe to conclude that succinate is oxidized mainly if not entirely to fumarate and malate. Nossal *et al.* (1956*a*) found that fractions from yeast capable of oxidizing several substrates oxidized malate poorly, if at all.

The P/O value with 2-deoxyglucose is higher than with glucose (Table 8), a consistent finding with sucrose-washed granules which supports the idea that glucose 6-phosphate is further metabolized. On the basis of the glucose 6-phosphate changes in the presence and absence of succinate, it can be calculated that 1·37 μmoles of glucose 6-phosphate were formed in the succinate experiment. This value is in good agreement with the P_i uptake of 1·21 μmoles, although this calculation rests on the assumption that glucose 6-phosphate utilization is unaffected by the presence of succinate.

By using particles prepared by washing and suspending in NaCl rather than sucrose, it has been possible to demonstrate more clearly that glucose 6-phosphate is the major phosphorylated product arising from phosphate as a result of succinate oxidation (Table 9). The changes in oxygen uptake, P_i, and glucose 6-phosphate in the presence and absence of succinate are shown. Considerable glucose 6-phosphate was present at the start of the experiment even in the absence of

Table 8. *Products of the oxidation of succinate*

Particles were prepared from Red Star Yeast by 5 sec. disintegration and washed and suspended in sucrose–EDTA. Particles representing 3·5 mg. of protein were used in a total volume of 1·3 ml. When 2-deoxyglucose was used as the acceptor of P_i, hexokinase was dialysed free from glucose. All values represent changes during 30 min. reaction period.

Substrate	P_i acceptor	O_2 uptake (μg. atoms)	P_i uptake (μmoles)	P/O	Fumarate–malate produced (μmoles)	Glucose 6-phosphate produced (μmoles)
None	Glucose	0·2	0·05	0·25	—	−0·93
None	2-Deoxyglucose	0·1	0	0	—	—
Succinate	Glucose	2·35	1·21	0·51	1·64	0·44
Succinate	2-Deoxyglucose	2·2	1·67	0·75	1·82	—

substrate because of the prior action of hexokinase on glucose and ATP. The amount of glucose 6-phosphate increased only slightly in the absence of succinate during the experimental period, indicating that no significant correction for myokinase activity is required. When succinate was present, glucose 6-phosphate increased by 1·41 μmoles during the experimental period as compared with 1·98 μmoles of P_i utilized. These results show the relative stability of glucose 6-phosphate with particles prepared in NaCl in contrast with particles washed and suspended in sucrose (Table 8). This result agrees with other evidence that particles prepared in NaCl are relatively free from pyridine nucleotide dehydrogenases.

It was shown in Table 1 that the absence of hexokinase markedly decreased phosphorylation. The experiments of Table 10 show that both hexokinase and glucose must be present for phosphorylation to occur to any significant degree. The residual phosphorylation observed in the absence of hexokinase or glucose may be due to small amounts of hexokinase and P_i acceptors associated with particles which have been washed only once. The glucose 6-phosphate formed in this experiment is given for purposes of comparison with Table 9.

The combination of observations that the oxidation of succinate is mainly a one-step reaction, that phosphorylation is dependent on the presence of succinate, glucose and hexokinase, and that glucose 6-phosphate is the major phosphorylated product indicate that, in the reaction under study, ATP is produced from P_i and ADP during the oxidation of succinate to fumarate. Since no substrate level phosphorylation is known for this oxidative step, the observations provide evidence independent of the 2:4-dinitrophenol experiments that the phosphorylation under study is associated with electron transport to oxygen.

Oxidation of other substrates. All the previous experiments have been concerned with the oxidation of succinate. Attempts have been made to substitute other substrates including lactate, α-oxoglutarate, ethanol, *iso*citrate, β-hydroxybutyrate and malate. The situation is complicated by the fact that particles prepared from yeast by different procedures possess different abilities to oxidize various substrates. Nossal *et al.* (1956*a*) have found that particles obtained by the disruption of yeast in phosphate (pH 7·6) can oxidize ethanol, *iso*citrate, α-oxoglutarate and lactate, but not malate. For reasons discussed above, particles prepared in phosphate are not suitable for studies of oxidative phosphorylation. Nossal, Keech & Utter (1956*b*) found that particles prepared by disruption in NaCl and washing in sucrose, such as have been used in this study, are also able to oxidize ethanol, *iso*citrate, α-oxoglutarate and lactate when appropriate cofactors are provided, but not malate or β-hydroxybutyrate. Some phosphorylation accompanied these oxidation reactions. However, the results of these experiments are highly variable and generally suggest that factors necessary for oxidation are retained to varying degrees in different batches of particles. Particles prepared by washing and suspending in NaCl oxidize only succinate and lactate.

It may be inferred that the particles prepared in phosphate have various dehydrogenase activities and probably contain other factors necessary for

Table 9. *Formation of glucose 6-phosphate*

Particles were prepared from Red Star Yeast by 5 sec. disintegration and washed and suspended in NaCl–EDTA. Approx. 4·5 mg. of protein was used in a total volume of 1·3 ml. The difference in the concentration of P_i in the two zero-time flasks is due to phosphorylation occurring in the flask with substrate during the equilibration period.

Substrate	Incubation time (min.)	O_2 uptake (μg. atoms)	P_i (μmoles)	ΔP_i (μmoles)	Glucose 6-phosphate (μmoles)	ΔGlucose 6-phosphate (μmoles)	P/O
None	0	—	5·0	—	0·85	—	—
None	25	0·1	5·0	0	0·99	+0·14	0
Succinate	0	—	4·03	—	1·30	—	—
Succinate	25	2·89	2·05	−1·98	2·71	+1·41	0·69

Table 10. *Requirement for hexokinase and glucose*

Particles were prepared as described in Table 9 and assayed with succinate for 30 min. with 3 mg. of protein/vessel in a total volume of 1·3 ml. Hexokinase (dialysed) or glucose was omitted as indicated.

Components	O_2 uptake (μg. atoms)	P_i uptake (μmoles)	P/O	Glucose 6-phosphate produced (μmoles)
All	1·65	1·44	0·87	1·21
Hexokinase omitted	1·51	0·22	0·15	—
Glucose omitted	1·65	0·17	0·13	—
Hexokinase and glucose omitted	1·96	0·39	0·2	—

the oxidation of other substrates. These same dehydrogenases and other factors must be virtually absent from granules prepared in NaCl. The particles prepared in sucrose show properties somewhere between those of these two preparations. To test the hypothesis that particles prepared in NaCl are unable to oxidize a substrate such as *iso*citrate because of factors lost to the supernatant, the latter was treated with ammonium sulphate (80% saturation) to precipitate most of the protein, and the resultant precipitate was dialysed and tested for ability to restore the ability of particles prepared in NaCl to oxidize *iso*citrate (Table 11). In the presence of both supernatant protein and DPN, the oxidation of *iso*citrate surpassed that of succinate. Omission of the supernatant protein abolished the oxidation of *iso*citrate, supporting the idea that a protein factor such as the dehydrogenase is missing from the granules. Omission of DPN decreased the oxidation to about one-third but the phosphorylation was little affected, suggesting that externally reduced DPN can be oxidized by the granules but that the oxidation is not accompanied by phosphorylation. This observation is in agreement with studies of animal mitochondria by Lehninger (1953). In the presence of DPN and supernatant protein some endogenous phosphorylation was observed, although 2-deoxyglucose and dialysed hexokinase were employed in this experiment in an effort to reduce endogenous reactions.

In contrast with *iso*citrate and other substrates poorly oxidized by unsupplemented particles prepared in NaCl, lactate is oxidized readily (Table 12). Lactate oxidation with most preparations is comparable with the oxidation of succinate, and the P/O values are only slightly lower. The addition of supernatant protein has no effect on the oxidation of succinate, but may increase the rate of oxidation of lactate although the degree of stimulation varies.

Table 11. *Oxidation of* isocitrate

Particles were prepared as described in Table 9 and assayed with 4·5 mg. of protein/vessel in a total volume of 1·6 ml. Where indicated, the vessels contained 40 μmoles of *iso*citrate, 1·5 mg. of dialysed protein prepared from the supernatant fraction as described in the text, 0·2 μmole of DPN and 40 μmoles of succinate. 2-Deoxyglucose was substituted for glucose and hexokinase was dialysed free from glucose.

Substrate added	Supernatant protein	DPN	O_2 uptake (μg. atoms)	P_i uptake (μmoles)	P/O
None	+	–	0	−0·19	0
None	+	+	0·2	0·31	1·5
iso-Citrate	–	+	0·2	0·2	1·0
iso-Citrate	+	–	0·67	0·58	0·87
iso-Citrate	+	+	1·95	0·68	0·35
Succinate	+	–	1·58	1·14	0·72

Table 12. *Oxidation of lactate*

Particles and assay procedure were as described in Table 11 except that 10 μmoles of DL-lactate were added where indicated.

Substrate	Supernatant protein	O_2 uptake (μg. atoms)	P_i uptake (μmoles)	P/O
None	–	0	0·15	—
None	+	0	0	0
Lactate	–	2·01	1·30	0·65
Lactate	+	2·72	1·60	0·59
Succinate	–	1·93	1·43	0·74
Succinate	+	1·96	1·43	0·73

Table 13. *Uncoupling by* 2:4-*dinitrophenol of phosphorylation during oxidation of lactate and succinate*

Particles and assay were as described in Tables 11 and 12 except that glucose was used as trapping system and no supernatant protein was added. 2:4-Dinitrophenol was added as indicated.

Substrate	2:4-Dinitrophenol (mM)	O_2 uptake (μg. atoms)	P_i uptake (μmoles)	P/O	Inhibition of P/O by 2:4-dinitrophenol (%)
Lactate	0	2·24	1·52	0·68	—
Lactate	0·1	1·93	0·95	0·49	28
Lactate	0·36	2·21	0	0	100
Succinate	0	2·45	2·13	0·87	—
Succinate	0·1	2·32	1·37	0·59	32
Succinate	0·36	2·03	0·08	0·04	95

The phosphorylation associated with the oxidation of lactate has a sensitivity to 2:4-dinitrophenol which corresponds closely to that associated with succinate oxidation (Table 13). Uncoupling is about 30 % for both substrates at a concentration of 0·1 mM-2:4-dinitrophenol and is essentially complete at 0·36 mM.

DISCUSSION

The present experiments establish that yeast cells contain sedimentable subcellular units able to couple phosphorylation with respiration. This finding strengthens the hypothesis that yeast cells contain organized respiratory particles similar to those found in animal and plant cells. The nature of the elements responsible for oxidative phosphorylation has not yet been established, although a major portion of the active material sediments in 15 min. at centrifugal forces low enough to suggest that a considerable degree of organization may exist in the particulate fraction. The findings that the yeast particulates are able to carry out oxidative phosphorylation without supplementation by soluble components of the cell, and that the yeast system is completely uncoupled by 2:4-dinitrophenol ally the yeast particles more with animal mitochondria than with microbial systems described previously.

The most obvious explanation of the rather low P/O values obtained for succinate oxidation by yeast granules is that the particles have been damaged during isolation and are therefore not exhibiting optimal phosphorylation. An alternative explanation is that the P/O value for succinate oxidation in yeast is 1 rather than 2. In a large number of experiments with a wide variety of preparative and assay procedures, very few P/O values have exceeded 1; further, a small part of the observed phosphorylation may not be linked to respiration. It is interesting to recall that Slater (1953) proposed that the P/O value for succinate oxidation in insect muscle was 1.

The failure in the present experiments to demonstrate an obligatory coupling of oxidation and phosphorylation can also be explained by assuming that the particles are not isolated in their native state, but the possibility must also be considered that no obligatory coupling exists in yeast.

It is possible that the yeast particles represent a mixture of active and inactive units. This would explain the low P/O values but it is difficult to see why such a mixture would not show some degree of obligatory coupling of oxidation and phosphorylation unless there is interaction between the two species of particles.

Phosphorylation associated with a flavin-linked lactate oxidation has not been described previously. Lactic dehydrogenase in yeast has been shown by Bach, Dixon & Zerfas (1946) and Appleby & Morton (1954) to be a flavo-haemoprotein with many properties analogous to the succinic dehydrogenase of heart. When considered together with the present studies, the presence in yeast of a lactoxidase unit as well as a succinoxidase unit can be postulated.

SUMMARY

1. Washed particulate fractions obtained by differential centrifuging from baker's yeast disintegrated by high-speed reciprocal shaking with glass beads show phosphorylation coupled to the oxidation of succinate and lactate.

2. Phosphorylation is dependent on the presence of an adenosine triphosphate-trapping system, yields glucose 6-phosphate as the major product and is completely uncoupled by 2:4-dinitrophenol.

3. Adenosine diphosphate is the only substance tested capable of acting as a phosphate acceptor; the other nucleoside diphosphates and adenosine monophosphate are completely inactive.

4. The P/O values observed during succinate oxidation generally range from 0·6 to 0·9 and phosphorylation is not obligatorily coupled to respiration.

5. Succinate is oxidized mainly or entirely to fumarate and malate.

6. The oxidation of substrates other than succinate or lactate is dependent upon the nature of the disintegration procedure and subsequent isolation procedure.

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Metabolism of Polycyclic Compounds

12. AN ACID-LABILE PRECURSOR OF 1-NAPHTHYLMERCAPTURIC ACID AND NAPHTHOL: AN *N*-ACETYL-*S*-(1:2-DIHYDROHYDROXYNAPHTHYL)-L-CYSTEINE*

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The presence of acid-labile precursors of mercapturic acids in the urines of animals dosed with bromobenzene and chlorobenzene was first noticed by Baumann & Preusse (1879) and Jaffe (1879). In studying the metabolism of naphthalene in rats and rabbits, Boyland & Wiltshire (1953) observed a metabolite in the urine which gave the colour reactions of 1-naphthol, but which was not extracted from neutral solution by ether. This so-called 'ether-insoluble 1-naphthol' was not present in the urine of animals dosed with 1-naphthol. In these experiments the excreted 1-naphthol was estimated colorimetrically, either by coupling with diazotized *p*-nitroaniline or by interaction with 2:6-dichloroquinonechloroimide. In both methods of estimation the urine was acidified before the addition of the reagents.

In a further investigation Boyland & Solomon (1956) estimated a number of the metabolites of naphthalene by measurement of the areas of the spots produced by the various metabolites on paper chromatograms. In the course of this work a method was developed for the determination on chromatograms of 1-naphthylmercapturic acid (I), first isolated by Bourne & Young (1934), depending on the pink fluorescence seen in ultraviolet light in the presence of ammonia. It was noticed that the urines of rats and rabbits treated with naphthalene contained little or no 1-naphthylmercapturic acid, but that after acidification of the urines the acid was liberated from a precursor (Solomon, 1954).

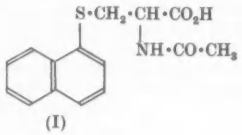
Knight & Young (1957) have recently detected the presence of acid-labile mercapturic acid precursors (for which they propose the name premercapturic acids) in the urine of animals after the administration of benzene, anthracene, fluorobenzene, chlorobenzene, bromobenzene and iodobenzene, as well as with naphthalene. They also noticed that benzyl chloride was excreted directly as the corresponding mercapturic acid after administration to animals.

It has now been shown (Boyland, Sims & Solomon, 1957) that the 'ether-insoluble 1-naphthol' and the acid-labile precursor of 1-naphthylmercapturic acid are the same substance, treatment of the urine with mineral acid giving

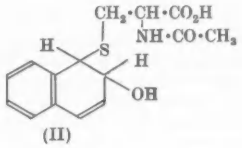
* Part 11: Boyland & Sims (1957).

rise to the mercapturic acid and to 1- and 2-naphthol. The present paper describes the isolation and properties of the precursor.

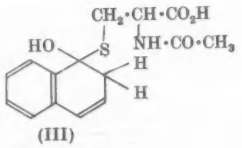
From a consideration of the evidence presented below it seems likely that the precursor is *N*-acetyl-*S*-(1:2-dihydro-2-hydroxy-1-naphthyl)-L-cysteine (II).



(I)



(II)



(III)

EXPERIMENTAL

Melting points are uncorrected.

Materials. *S*-1-Naphthyl-L-cysteine and 1-naphthylmercapturic acid were prepared from 1-naphthylamine by the method of du Vigneaud, Wood & Binkley (1941) as needles from aqueous ethanol, m.p. 182–183° and 169–170° respectively. 2-Naphthylmercapturic acid was the gift of Mr R. H. Knight. *N*-Acetylcysteine formed prisms from water, m.p. 110°, when prepared by the method of Pirie & Hele (1933). *NN'*-Diacetylcystine was prepared as described by Hollander & du Vigneaud (1931), as a colourless powder which became gummy on keeping. *trans*-1:2-Dihydro-2-hydroxy-1-naphthyl glucosiduronic acid was isolated from the urine of rabbits dosed with naphthalene (E. Boyland & P. Sims, unpublished work). 1-Naphthyl glucosiduronic acid was isolated from the urine of rabbits dosed with 1-naphthol by the method of Berenbom & Young (1951).

Paper chromatography. Whatman no. 1 chromatography paper was used throughout. Descending chromatograms were developed with either solvent system 1 or 2 for 15 hr. and ascending chromatograms with solvent system 3 for 5 hr. (see Table 1). After development the chromatograms were dried at room temperature and examined under u.v. light after exposure to NH_3. The chromatograms were then usually sprayed with a solution of freshly diazotized *p*-nitroaniline (0·2% in 0·1N-HCl), allowed to dry at room temperature and sprayed with aq. 10% Na_2CO_3. Occasionally the chromatograms were sprayed with diazotized sulphanilic acid (0·05% in 0·1N-HCl) or with a saturated solution of 2-chloro-4-nitrobenzenediazonium naphthalene-2-sulphonate [N.N.C.D. reagent (Boyland & Sims, 1953)] in 0·1N-HCl. In each case the air-dried chromatograms were then treated with aq. 10% Na_2CO_3. The properties on paper chromatograms of the compounds mentioned in this paper are summarized in Table 1.

Table 1. *Paper-chromatographic properties of compounds related to the* 1-*naphthylmercapturic acid precursor*

Solvent systems: 1, butanol saturated with aq. 2N-NH_3 (downward development); 2, the organic phase of a mixture of butanol–ethanol–water (17:3:20, by vol.) (downward development); 3, aq. 0·1N-NH_3 (upward development). The R_F values refer to the pure compounds. R_F values of the compounds detected in urine are usually slightly higher.

Metabolite	R_F in solvent 1	2	3	Fluorescence	Diazotized *p*-nitroaniline	Diazotized sulphanilic acid	N.N.C.D. reagent
1-Naphthylmercapturic acid precursor*	0·22	0·38	0·81	Dark-absorption	Blue	Red	Blue
1-Naphthylmercapturic acid*	0·37	0·35	0·69	Orange or pink†	—	—	—
2-Naphthylmercapturic acid*	0·36	0·36	0·72	Orange or pink†	—	—	—
1-Naphthol	0·96	0·96	0·62	Blue†	Blue	Red	Blue
2-Naphthol	0·96	0·96	0·55	Violet†	Orange	Orange	Orange
N-Acetylcystine*‡	0·01	—	0·86	Dark-absorption	—	—	—
NN'-Diacetylcystine*	0·01	—	0·86	Dark-absorption	—	—	—
1-Naphthyl glucosiduronic acid	—	—	0·80	Dark violet†	—	—	—

* Also detected by the platinic iodide reagent of Toennies & Kolb (1951) and by spraying with 1% $K_2Cr_2O_7$ in 5% aqueous acetic acid followed by saturated aqueous silver acetate (Knight & Young, 1957).

† After exposure to NH_3 vapour.

‡ After chromatography the compound failed to react immediately with the sodium nitroprusside reagent of Toennies & Kolb (1951) and is presumed to have become oxidized to *NN'*-diacetylcystine.

Dosing of animals and collection of urine. Six rabbits (body wt. approx. 2 kg.), maintained on oats, rat cake, cabbage and water, were each injected intraperitoneally on five consecutive days with a solution of naphthalene in arachis oil (5 ml. of 20%, w/v). The rabbits and other animals were housed in metabolism cages designed for the collection of urine separately from faeces. The urines were collected up to the sixth day and were stored at 0°.

Paper-chromatographic examination of the rabbit urines. Urines were examined by means of paper chromatography with the solvent systems described in Table 1, both before and after the addition of 10 N-HCl. Inspection of the papers showed that fresh urine contained the 1-naphthylmercapturic acid precursor, but no detectable amount of 1-naphthylmercapturic acid, whereas the acidified urine contained 1-naphthylmercapturic acid but no 1-naphthylmercapturic acid precursor. If the urines were kept either at room temperature or at 0°, detectable amounts of 1-naphthylmercapturic acid were present after a few days. Examination of the various solutions obtained during the working-up procedures described below showed that decomposition of the precursor was continually occurring, with the formation of 1-naphthylmercapturic acid and 1- and 2-naphthol.

Isolation of the 1-naphthylmercapturic acid precursor. The pooled urines were centrifuged to removed solid matter and acidified to pH 4 with acetic acid. Activated charcoal (500 g.) was added and the mixture was stirred for about 10 min. The charcoal was filtered off and washed with water (about 10 l.) to remove urea and inorganic salts, then with aq. 2 N-NH_3 (300 ml.), and finally with hot methanol containing benzene (5%, v/v) until most of the naphthalene metabolites had been eluted (about 10 l. of the solvent mixture was usually required). The methanol–benzene eluates were evaporated to dryness under reduced pressure to a brown gum. The material eluted from the charcoal by aq. 2 N-NH_3 contained some naphthalene metabolites together with large amounts of naturally occurring compounds, including allantoin, and was discarded.

A column was prepared by dry-packing cellulose powder (600 g., Whatman standard grade) into a glass chromatography column (100 cm. × 7 cm.) by the method of Hough, Jones & Wadman (1949). A mixture of butanol–*cyclo*hexane–aq. 2 N-NH_3 (2 l. of 9:2:1, by vol.) was passed through the column. The gum containing the naphthalene metabolites was dissolved in a small volume of methanol and the solution was adsorbed on cellulose powder (30 g.) which was packed in a layer on top of the cellulose in the column. The column was developed with the above solvent mixture and the eluate was collected in fractions of about 500 ml. volume.

A few drops of aq. NH_3 (sp.gr. 0·88) were added to the fractions, which were then separately evaporated to volumes of about 20 ml. under reduced pressure. The solutions thus obtained were examined by means of paper chromatography with the solvent systems described in Table 1. It was not possible to separate completely the naphthalene metabolites from one another on a column as described above; nor was it possible to reproduce absolutely the chromatographic conditions. All the known metabolites of naphthalene were, however, detected in one or more of the fractions, by methods already published (e.g. Boyland & Solomon, 1956). The results obtained from a typical separation are shown in Table 2.

The fractions containing the 1-naphthylmercapturic acid precursor were combined and rechromatographed on a smaller cellulose-powder column (prepared in the manner already described from about 150 g. of powder), fractions of about 200 ml. being collected. The fractions in which the 1-naphthylmercapturic acid precursor was detected also contained at least two unknown phenolic substances and gave positive Tollens tests for glucuronide; naphthalene, as well as 1-naphthylmercapturic acid, was liberated after acidification of the fractions with HCl. These fractions were combined and, after the addition of a little aq. NH_3 (sp.gr. 0·88), evaporated to about 10 ml. under reduced pressure. When the solution was kept at 0°, the ammonium salt of the 1-naphthylmercapturic acid precursor separated, and was filtered off and washed with a few drops of ice-cold ethanol. The mother liquors were kept at 0° in an open

Table 2. *Naphthalene metabolites eluted from a typical cellulose powder column with butanol–cyclohexane–2 N-NH_3 (9:2:1, by vol.)*

Fractions of 500 ml. were collected and examined by paper chromatography (see Table 1). 1-Naphthol was detected in all the fractions up to no. 12. A number of substances giving red or purple colours with diazotized *p*-nitroaniline were present in all the fractions; these are presumed to be naturally occurring phenols.

Fraction no.	Metabolite
1, 2	1- and 2-Naphthol
3	*trans*-1:2-Dihydro-1:2-dihydroxynaphthalene
4, 5, 6	1-Naphthyl sulphate, 2-hydroxy-1-naphthyl sulphate and an unknown*
7, 8	The compounds in fractions 4, 5 and 6 and 1-naphthylmercapturic acid
9	1-Naphthylmercapturic acid precursor and an unknown†
10, 11, 12	1-Naphthylmercapturic acid precursor
13	—
14, and later fractions	1-Naphthyl glucosiduronic acid, 1:2-dihydro-2-hydroxy-1-naphthyl glucosiduronic acid and two unidentified substances‡

* This substance (R_F 0·53 in solvent 1) gave an immediate blue colour with diazotized *p*-nitroaniline and an immediate purple colour with this reagent when the chromatogram was sprayed with 2 N-HCl and heated at 70° for 20 min. It is believed to be 1-hydroxy-2-naphthyl sulphate.

† This substance (R_F 0·24 in solvent 1) gave a blue colour with diazotized *p*-nitroaniline, and on two-dimensional chromatograms which were sprayed with 2 N-HCl between the first and second runs gave rise to 1-naphthol and a compound which was either 1- or 2-naphthylmercapturic acid. The substance was possibly *N*-acetyl-*S*-(1:2-dihydro-1-hydroxy-2-naphthyl)-L-cysteine.

‡ These two substances (R_F 0·16 and 0·05 in solvent 1) gave blue and yellow colours repectively with diazotized *p*-nitroaniline. After the chromatograms were treated with 2 N-HCl as described above, both substances gave purple colours with diazotized *p*-nitroaniline. They are thought to be 1-hydroxy-2-naphthyl and 2-hydroxy-1-naphthyl glucosiduronic acid respectively.

vessel for several days, when more of the precursor crystallized. When no more crystals separated from the mother liquors (paper chromatography showed that the precursor was slowly decomposing into 1-naphthylmercapturic acid and 1- and 2-naphthol), the liquors were evaporated under reduced pressure and rechromatographed on a fresh cellulose-powder column. By this means the ammonium salt of the precursor was obtained in yields of between 300 and 900 mg. from each batch of urine collected from rabbits dosed with 30 g. of naphthalene.

Properties of the 1-naphthylmercapturic acid precursor. The ammonium salt obtained above was recrystallized from propanol in needles, m.p. 142° (decomp.), which were dried at 100°/0·5 mm. for 15 min., to yield the ammonium salt of *N-acetyl*-S-(1:2-*dihydro*-2-*hydroxy*-1-*naphthyl*)-L-*cysteine* (II) (Found: C, 55·8; H, 6·5; N, 8·1; S, 9·9. $C_{15}H_{20}O_4N_2S$ requires C, 55·55; H, 6·2; N, 8·6; S, 9·9%). Crystals dried at 25°/0·5 mm. for 2 hr. apparently contained propanol of crystallization (Found: C, 56·3; H, 7·2. Calc. for $C_{15}H_{20}O_4N_2S,C_3H_8O$: C, 56·2; H, 7·3%). If the compound was heated at 100°/0·5 mm. for periods of more than 30 min. decomposition occurred, and it was shown by paper chromatography that the gum thus formed contained 1-naphthylmercapturic acid and 1- and 2-naphthol. When the ammonium salt was separated from methanol by means of ether, the product formed needles, m.p. 142° (decomp.), and appeared to contain ether of crystallization (Found, after drying at 25°/0·5 mm.: C, 56·8, 56·7; H, 7·0, 7·2; N, 7·6; S, 8·4. Calc. for $C_{15}H_{20}O_4N_2S,\frac{1}{2}C_4H_{10}O$: C, 56·5; H, 7·0; N, 7·75; S, 8·9%). Similarly, from methanol–benzene the product formed needles, m.p. 142° (decomp.), which appeared to contain benzene of crystallization (Found, after drying at 25°/0·5 mm. for 3 hr.: C, 59·2; H, 6·3; N, 7·5; S, 8·8. Calc. for $C_{15}H_{20}O_4N_2S,\frac{1}{2}C_6H_6$: C, 59·5; H, 6·4; N, 7·7; S, 8·8%). When the samples crystallized from the last two solvent mixtures were heated to above 70° at 0·5 mm. for more than 15 min. decomposition occurred.

The product obtained by crystallization from methanol–benzene had (after drying at 25°/0·5 mm.) $[\alpha]_D^{22} - 338 \pm 2°$ in water (*c*, 0·5) and the u.v. absorption, measured in water, showed $\lambda_{max.}$ 258 mμ and $\epsilon_{max.}$ 6310. The infrared spectrum, measured as a mull in liquid paraffin, showed a band at 800 cm.$^{-1}$, whereas the infrared spectrum of 1-naphthylmercapturic acid itself has two bands, at 771 and 798 cm.$^{-1}$. It is probable that the simplification of these bands in the precursor is due to the reduction of one of the rings of the naphthalene nucleus in this compound.

Decomposition of the 1-naphthylmercapturic acid precursor by acid. When the ammonium salt of the precursor (50 mg.), in water (0·5 ml.), was treated with a few drops of 10N-HCl, 1-naphthylmercapturic acid separated within a few seconds. After 5 min. the crystals were filtered off and recrystallized from aqueous ethanol in needles, m.p. 169°, undepressed in admixture with samples prepared (*a*) from the urine of rabbits injected with naphthalene by the method of Bourne & Young (1934), and (*b*) synthetically (Found: N, 4·8; S, 11·0. Calc. for $C_{15}H_{15}O_3NS$: N, 4·8; S, 11·1%). The presence of 1- and 2-naphthol in the mother liquors was demonstrated by means of paper chromatography with solvent 3.

In a second experiment a weighed amount of the ammonium salt (which had been recrystallized from methanol–benzene) was treated as before, and the precipitated 1-naphthylmercapturic acid was filtered off, washed with a little ice-cold water and weighed. The combined filtrate and washings were extracted with ether (3 × 2 ml.) and the ethereal solution was applied along the base line of chromatography paper and developed upward with solvent 3 (Table 1) for 6 hr. The paper was dried and the positions of the 1- and 2-naphthol bands determined under u.v. light. These bands were cut out and eluted separately with ethyl acetate (3 × 3 ml.). The solutions were each made up to 10 ml. with ethyl acetate and 1- and 2-naphthol determined on a Unicam SP. 500 spectrophotometer with 2:6-dichloroquinonechloroimide reagent (Gibbs, 1927), the colours being measured at 605 mμ. Standard curves were prepared by chromatographing and eluting known amounts of the naphthols. The results of duplicate determinations are shown in Table 3.

The aqueous layers from the ether extractions were spotted on paper chromatograms, which were developed with solvent 3 (Table 1). A compound was detected with the $K_2Cr_2O_7$–silver acetate reagent which had R_F 0·86 and did not give an immediate colour with the sodium nitroprusside reagent of Toennies & Kolb (1951). When chromatographed under these conditions, *N*-acetylcysteine also failed to give a colour with sodium nitroprusside (see Table 1).

The precursor (10 mg.), in water (0·2 ml.), was also treated with 10N-H_2SO_4, with 10N-H_3PO_4 and with dry methanol saturated with HCl (0·2 ml. of each reagent): in each case immediate decomposition occurred, 1-naphthylmercapturic acid and 1- and 2-naphthol being detected in the products by paper chromatography. When the precursor was similarly treated with acetic acid no decomposition was detected within 24 hr.

Table 3. *Acid decomposition of the 1-naphthylmercapturic acid precursor*

Precursor (assumed to be $C_{15}H_{20}O_4N_2S,\frac{1}{2}C_6H_6$) (mg.)	1-Naphthylmercapturic acid (mg.)	1-Naphthol (mg.)	2-Naphthol (mg.)
25·0	17·2 (86·5%)	0·09	0·18
24·6	16·8 (85·5%)	0·06	0·13

Table 4. *Acid decomposition of* trans-1:2-*dihydro*-2-*hydroxy*-1-*naphthyl glucosiduronic acid*

Treatment	1-Naphthol	2-Naphthol	1-Naphthyl glucosiduronic acid
Kept at 25° for 24 hr.	–	Trace	+
Heated at 60° for 10 min.	–	+	+ +
Heated at 100° for 30 min.	+ +	+	–

Decomposition of trans-1:2-*dihydro*-2-*hydroxy*-1-*naphthyl glucosiduronic acid by acid.* The glucosiduronic acid (15 mg.) in 2 N-HCl (0·2 ml.) was treated as shown in Table 4 and the products were examined by means of paper chromatography with solvent 3 (Table 1).

Decomposition of the 1-*naphthylmercapturic acid precursor with alkali.* The ammonium salt of the precursor (50 mg.) was heated to 100° with aqueous N-NaOH (1 ml.) for 5 min. Ammonia was evolved and examination of the products on paper chromatograms showed the presence of 1-naphthylmercapturic acid, 2-naphthol and a substance [which could only be detected when solvent 3 (Table 1) was used] which was presumed to be 1-thionaphthol. This substance was also present in the products from the alkaline hydrolysis (under similar conditions) of 1-naphthylmercapturic acid itself. 1-Naphthol was not detected in the products of the alkaline hydrolysis of the precursor.

When the ammonium salt (50 g.) was heated at 100° with N-NaOH (1 ml.) for 45 min. and the solution diluted and aerated as described by Bourne & Young (1934), then 1:1-dinaphthyl disulphide (15 mg.), m.p. 88–89°, was obtained. Bourne & Young obtained a similar product from the alkaline hydrolysis and oxidation of 1-naphthylmercapturic acid.

Paper chromatography showed that, when the ammonium salt of the precursor was kept overnight at room temperature with aq. N-NaOH, some decomposition with the formation of 1-naphthylmercapturic acid and 2-naphthol occurred. On the other hand, a solution of the ammonium salt in pyridine at room temperature was stable for at least 48 hr. Attempts to prepare the sodium and potassium salts of the precursor from the ammonium salts by the addition of one equivalent of alkali resulted in the formation of gums, in which the presence of the breakdown products of the precursor was demonstrated.

Action of silver salts on the 1-*naphthylmercapturic acid precursor.* The work of Peters & Wakelin (1947) suggested that it might be possible to split the thio ether linkage of the precursor to yield a 1:2-dihydro-1:2-dihydroxynaphthalene. The precursor (10 mg.) in water (0·2 ml.) and 10% aq. $AgNO_3$ (0·05 ml.) was treated with 0·1 N-NaOH (0·5 ml.). The solution was filtered and the filtrate extracted with ether (2 × 1 ml.). The aqueous and ether layers were examined on paper chromatograms, revealing 1- and 2-naphthol and 1-naphthylmercapturic acid. No diol was detected. The precursor (in 10 mg. portions) in water (0·2 ml.) was also treated with 10% aq. $AgNO_3$ (0·05 ml.) alone, saturated aqueous silver acetate (0·1 ml.) and freshly prepared Ag_2O (0·1 g.). In each experiment the products, identified on paper chromatograms, were 1- and 2-naphthol and 1-naphthylmercapturic acid.

Attempted preparation of derivatives of the 1-*naphthylmercapturic precursor.* (*a*) The methyl ester. The ammonium salt of the 1-naphthylmercapturic acid precursor (100 mg.) was suspended in ether and treated with an excess of diazomethane in ether. The compound slowly dissolved with evolution of gas. When the reaction had finished the ether was distilled off, leaving a colourless gum which could not be crystallized. Examination of the gum on paper chromatograms showed the presence of a little 2-naphthol, together with a substance which had R_F 0·85 in solvent 1 (Table 1), and which showed as a dark absorbent region in ultraviolet light and gave a blue colour with diazotized *p*-nitroaniline and Na_2CO_3. These are the properties expected of the methyl ester of the precursor.

(*b*) The *O*-acetyl derivative. The ammonium salt of the precursor (100 mg.), in pyridine (1 ml.), was treated with acetic anhydride (0·5 ml.) at 0° for 5 hr. The yellow solution was poured into water and the mixture extracted with ether (3 × 50 ml.). The ether was evaporated, leaving a clear gum which could not be crystallized. The gum was dissolved in a little saturated aq. $NaHCO_3$ and the solution was washed with ether, acidified with conc. HCl and extracted with ether. The ether was evaporated and the residual gum examined by paper chromatography both before and after heating with 5 N-HCl for 15 min. 1-Naphthylmercapturic acid and a trace of 2-naphthol were detected in the acid-treated material.

Detection of the 1-*naphthylmercapturic acid precursor in species other than rabbit.* The animals listed in Table 5 were dosed with a solution of naphthalene in arachis oil (20%, w/v) as shown in the table. The urines produced in the 24 hr. after treatment were examined on paper chromatograms before and after acidification with HCl. For the examination of the human urine, the metabolites were first concentrated by absorption on charcoal and elution as described above. Estimates of the amounts of the mercapturic acid and its precursor were obtained by a comparison of the sizes of the spots produced with those of the spots produced by the other known naphthalene metabolites.

Table 5. *Formation of* 1-*naphthylmercapturic acid and its precursor in different species*

The rats were dosed orally, the other animals by intraperitoneal injection. The human subjects were dosed orally.

Species	Dose (naphthalene in arachis oil; 20%, w/v) (g.)	1-Naphthylmercapturic acid precursor	1-Naphthylmercapturic acid	
			Before acidification of the urine	After acidification of the urine
Rabbit	1·0	+ +	–	+ +
Rat (male)	0·1	+ +	–	+ +
Rat (female)	0·1	+ +	–	+ +
Mouse	0·02	+ +	Trace	+ +
Hamster	0·1	+ +	–	+ +
Guinea pig	0·4	+	–	+
Man 1	0·5	Trace	–	Trace
Man 2	0·5	Trace	–	Trace
Man 3	0·5	+	–	+

Examination of possible intermediates in the formation of the 1-naphthylmercapturic acid precursor in the rabbit. *S*-1-Naphthyl-L-cysteine (0·5 g. in arachis oil), (±)-*trans*-1:2-dihydro-1:2-dihydroxynaphthalene (1 g. in arachis oil), 1-naphthol (1 g. in arachis oil), 2-naphthol (0·5 g. in arachis oil) and 1:2-dihydronaphthalene (0·5 ml.) were each injected intraperitoneally into rabbits and the urines collected for 24 hr. after injection. The urines were examined by means of paper chromatography both before and after acidification with 10 N-HCl. With *S*-1-naphthyl-L-cysteine, 1-naphthylmercapturic acid was detected in the urine both before and after acidification, whereas none was present in the urines (either before or after acidification) from the animals treated with (±)-*trans*-1:2-dihydro-1:2-dihydroxynaphthalene or with 1- or 2-naphthol. None of these urines contained the 1-naphthylmercapturic acid precursor. A trace of the 1-naphthylmercapturic acid precursor was found in the unacidified urine and of 1-naphthylmercapturic acid in the acidified urine of the animal treated with 1:2-dihydronaphthalene.

DISCUSSION

The 1-naphthylmercapturic acid precursor represents a new type of metabolite of aromatic compounds, although the earlier work of Baumann & Preusse (1879) and Jaffe (1879) suggested that acid-labile precursors of the mercapturic acids of the halogenated benzenes were formed when the halogenobenzenes were fed to animals. The work of Knight & Young (1957) and experiments with bromobenzene and anthracene (E. Boyland & P. Sims, unpublished observations) suggest that the formation of these precursors is a general metabolic pathway of aromatic compounds in animals. The apparent direct combination of benzyl chloride (Knight & Young, 1957) and some chloronitrobenzenes (Bray, James & Thorpe, 1955*a*, *b*, 1956; Bray & James, 1957) with cysteine (with elimination of chlorine) in the body might well be due to the presence of reactive chlorine atoms in these compounds.

The structure of the 1-naphthylmercapturic acid precursor has not yet been unequivocally established. The extreme ease with which the compound breaks down has made the preparation of derivatives difficult. The fact that the compound is decomposed immediately by mineral acids suggests a 1:2-dihydrohydroxynaphthalene type of structure, similar to that of 1:2-dihydro-1-naphthyl glucosiduronic acid (Boyland & Solomon, 1955), which is immediately decomposed by acid to yield naphthalene. The 1:2-dihydronaphthalene structure is supported by the light-absorption data (see Fig. 1). The spectrum of the precursor resembles that of 1:2-dihydronaphthalene rather than that of 1:4-dihydronaphthalene. The two most likely structures for the precursor are (II) or (III) above. Both of these compounds would be expected to lose the elements of water readily, to give 1-naphthylmercapturic acid. The simultaneous formation of naphthols suggests that elimination of *N*-acetylcysteine can also take place. We have not detected this compound in the decomposition products, but there is evidence for the presence of *NN'*-diacetylcysteine, which may have been produced from *N*-acetylcysteine during the paper chromatography. Knight & Young (1957), using animals treated with ^{35}S-labelled cysteine as well as with naphthalene, found that a ^{35}S-labelled compound other than 1-naphthyl[^{35}S]mercapturic acid was produced when the precursor was decomposed by acid. The formation of both 1- and 2-naphthol from the precursor on acidification is difficult to explain by assuming the existence of either structure (II) or (III). There is possibly an acid-induced migration of a hydroxyl radical, but in the analogous case of 1:2-dihydro-2-hydroxy-1-naphthyl glucosiduronic acid decomposition with cold mineral acid occurs, at a very much slower rate, with the formation of 1-naphthyl glucosiduronic acid and 2-naphthol; no 1-naphthol has been detected. The formation of 1-naphthylmercapturic acid and 2-naphthol by the action of alkali on the precursor can be readily explained if the precursor has structure (II). The breakdown of the precursor by either acid or alkali gives rise mainly to 1-naphthylmercapturic acid; the formation of the naphthols and *N*-acetylcysteine is

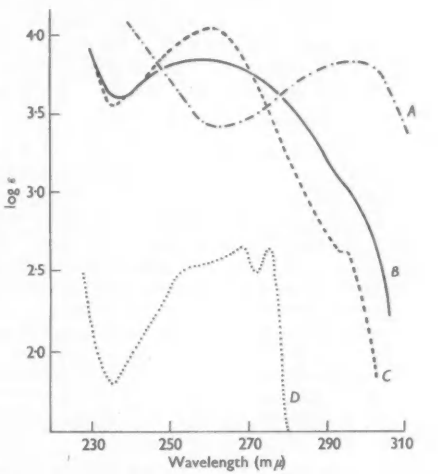


Fig. 1. Ultraviolet-absorption spectra of 1-naphthylmercapturic acid (*A*), the 1-naphthylmercapturic acid precursor (*B*), 1:2-dihydronaphthalene (*C*) and 1:4-dihydronaphthalene (*D*). (*A*) was measured in aq. 0·1 N-NH_3, (*B*) in water, and (*C*) and (*D*) in ethanol.

clearly a secondary reaction. The evidence so far obtained favours structure (II) for the precursor, but attempts to confirm this structure by the formation either of 2-naphthyl acetate on acetylation and acid decomposition or of a 1:2-dihydronaphthalene-1:2-diol by the action of silver salts have not been successful, the precursor being too readily decomposed by these reagents. A compound of structure (II) could exist in *cis* and *trans* forms, but there is no evidence as to which form the precursor possesses.

Baumann & Preusse (1879) and Jaffe (1879) found that the lead salts of the precursors of *p*-bromo- and *p*-chloro-benzenemercapturic acid possessed high optical rotations, whereas those of the mercapturic acids themselves are low. For the naphthalene derivatives the value of $[\alpha]_D^{22} - 338°$ of the precursor may be compared with that of $[\alpha]_D^{22} - 25°$ obtained by Bourne & Young (1934) for 1-naphthylmercapturic acid.

The mechanism by which the precursor arises in the body is not known. Of the possible intermediates tested, only 1:2-dihydronaphthalene gave rise to any detectable amount of precursor, and it is possible that the precursor arose from naphthalene produced by the dehydrogenation of the dihydro compound *in vivo*. It seems likely from previous work (e.g. du Vigneaud *et al.* 1941; West & Mathura, 1954) that the acetylation of the amino group occurs after the addition of cysteine (or a compound containing a cysteine residue) to the naphthalene nucleus. The formation of a compound such as (II) above would require the addition of cysteine together with a hydroxyl group to naphthalene, or the direct addition of the unknown cysteine sulphenic acid

$$(HO_2C \cdot CH(NH_2) \cdot CH_2 \cdot S \cdot OH).$$

The work of Stekol (1937), Gutmann & Wood (1950) and Mills & Wood (1956) suggests that with the halogenobenzenes reaction occurs between the aromatic compound and tissue-bound amino acid sulphur, whereas Barnes & James (1957) and Bray & Franklin (1957) favour reaction of the aromatic compound with the SH group of glutathione followed by hydrolysis with glutathionase. If either of these reactions occur with naphthalene, the reaction between the hydrocarbon and an S–OH group, rather than with an SH group, would seem to be necessary.

An alternative mechanism would be the reaction between the SH group of cysteine (or a tissue-bound or glutathione SH group) and the unknown naphthalene-1:2-epoxide. Such an intermediate has already been postulated (Boyland, 1950) to account for the formation of 1:2-dihydro-1:2-dihydroxynaphthalene in naphthalene-treated animals. From such a reaction the formation of the isomeric *N*-acetyl-*S*-(1-hydroxy-1:2-dihydro-2-naphthyl)-L-cysteine might be expected. We have detected a small amount of a substance in rabbit urine which might well be this compound (see Table 2). This substance and the postulated epoxide could be reduced in the body to 1:2-dihydro-1-hydroxynaphthalene, the glucosiduronic acid derivative of which has been described (Boyland & Solomon, 1956).

The 1-naphthylmercapturic acid precursor has been detected in the urine of all the species which we have examined, and it is probable that it also formed in the other species in which the formation of 1-naphthylmercapturic acid is known to occur.

SUMMARY

1. A substance has been detected in and isolated from the urine of rabbits dosed with naphthalene which on acidification with mineral acid gives rise to 1-naphthylmercapturic acid and 1- and 2-naphthol.

2. From a consideration of the acid decomposition products and the fact that it gives 1-naphthylmercapturic acid and 2-naphthol on treatment with alkali it is suggested that the compound is *N*-acetyl-*S*-(1:2-dihydro-2-hydroxynaphthyl)-L-cysteine.

3. A number of physical properties of the compound are recorded.

4. The compound was not produced when rabbits were injected with 1- and 2-naphthol, (±)-*trans*-1:2-dihydro-1:2-dihydroxynaphthalene or *S*-1-naphthyl-L-cysteine. Traces were produced with 1:2-dihydronaphthalene.

5. Rats, mice, hamsters, guinea pigs and man all excreted the precursor after treatment with naphthalene.

6. Possible modes of formation of the precursor in the body are discussed.

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The Preparation of Barium Monophosphotaurocyamine

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The synthesis and isolation of monophosphotaurocyamine (*N*-phosphoguanidoethanesulphonic acid) has not been described, although the compound is of interest as the naturally occurring phosphagen in certain annelids (van Thoai, Roche, Robin & van Thiem, 1953). These authors reported its isolation as a calcium salt after the phosphorylation of taurocyamine by phosphoryl chloride in an alkaline medium. A later communication (van Thoai & van Thiem, 1957*a*) reported the isolation of phosphotaurocyamine as a crystalline ammonium salt, but no details were given. In view of the difficulties which have been encountered by the present workers, because at least two products are formed in the phosphorylation reaction, it was considered worth while to report details of the synthesis and isolation.

This communication deals with the synthesis of taurocyamine, the products of the reaction between phosphoryl chloride and taurocyamine, the separation in pure form of barium monophosphotaurocyamine and some of its properties.

MATERIALS AND METHODS

Taurine (British Drug Houses Ltd.) was used without further purification.

Taurocyamine was estimated by the method described by Rosenberg, Ennor & Morrison (1956) for the determination of arginine. Bound taurocyamine was estimated by the same method after hydrolysis of the N–P bond in N-HCl at 100° for 10 min.

Inorganic phosphate was estimated by the method described by Ennor & Stocken (1950).

All hydrolyses and enzyme experiments were carried out with the sodium salts of monophosphotaurocyamine and fraction II (see later); these were prepared by passage of solutions of the barium salts through columns of Zeo-Karb 225 (Na^+ form).

Myosin was prepared by Dr H. Rosenberg of this Department from rabbit skeletal muscle as described by Bailey (1942). It was stored in 50% (v/v) glycerol at −10° and contained 2·5 mg. of protein/ml.

Taurocyamine phosphokinase (TPK) was purified from aqueous extracts of *Arenicola assimilis* by a method which will be described later and which involved fractionation with acetone, ammonium sulphate and ethanol. The enzyme was stable and was stored in 50% (v/v) glycerol at −10°; the solution contained 1·86 mg. of protein/ml.

The purified intestinal alkaline phosphatase was a gift from Professor R. K. Morton, University of Adelaide.

Taurocyamine was prepared by allowing *S*-methyl*iso*thiourea to react with taurine in an alkaline medium in the technique described by Schutte (1943) for the preparation of guanidines. Thus 72 g. of taurine was dissolved in 250 ml. of aq. 17N-NH_3 soln. and 120 g. of *S*-methyl*iso*thiourea sulphate added. The mixture was heated to 60° and stirred vigorously until the reactants dissolved. There was a vigorous evolution of methanethiol and the mixture was allowed to cool to room temperature. Crystallization of taurocyamine commenced after a few minutes and the reaction was allowed to proceed for 20 hr. The crystalline material was filtered off, washed with ice-cold water and

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the filtrate discarded. The solid material was recrystallized several times from water. Yield, 50 g.; m.p. 265° [cf. m.p. 228–230° (van Thoai & Robin, 1954); m.p. 264° (Dittrich, 1878); m.p. 228° (Engel, 1875)]. The purity of the material was checked by N analysis and paper chromatography.

RESULTS

Initial experiments. Taurocyamine was allowed to react with $POCl_3$ in an alkaline medium and the barium salt of the product isolated from the mixture. This salt was assumed to be the monophosphate, i.e. $C_3H_8O_6N_3SPBa$, but analysis of the material dried over P_2O_5 *in vacuo* at 100° showed Ba = 40·8 % (theory = 35·8 %) and P:taurocyamine ratio = 1·10. Two samples prepared subsequently gave P:taurocyamine ratios 1·33 and 1·44, indicating contamination by a compound containing P. Determinations were made of P and taurocyamine released from the compound during hydrolysis in N-HCl at 37°. There was a progressive decrease with time of the P:taurocyamine ratio from a maximum value of 1·81 (Table 1), which was interpreted as indicating the presence of a mono- and di-phosphotaurocyamine. As a consequence some of the material was placed on an anion-exchange resin (IRB-400, Cl^- form), from which two fractions were subsequently eluted: one with 0·1M-NaCl having a P:taurocyamine ratio = 1:1, the other with 2M-NaCl and a ratio = 2:1 (fraction II). The details of the synthesis and separation of the two phosphorylated derivatives are given below.

Table 1. *Hydrolysis of crude phosphotaurocyamine*

Crude phosphotaurocyamine (1·1 mM on the basis of taurocyamine) was hydrolysed in N-HCl at 37°.

Time (min.)	P (μmoles/ml.)	Taurocyamine (μmoles/ml.)	Molar ratio P:Taurocyamine
2·5	0·047	0·0215	1·81
5	0·075	0·038	1·75
10	0·127	0·07	1·69
40	0·406	0·250	1·59
50	0·458	0·302	1·48
120	0·845	0·588	1·42
180	1·04	0·755	1·36
	1·47*	1·09*	1·34*

* These results were obtained from a sample hydrolysed in N-HCl at 100° for 15 min.

Preparation of crude phosphotaurocyamine. Taurocyamine (20 g., 0·12 mole) was added to a three-necked flask (2 l.) containing 20 ml. of water and 50 ml. of 10N-NaOH. The mixture was stirred rapidly and 38 ml. (0·43 mole) of $POCl_3$ and 170 ml. of 10N-NaOH were added over a period of 1 hr. and at such a rate that the mixture remained strongly alkaline. The contents of the flask were maintained between −5° and 0° by periodic immersion in a solid CO_2–ethanol mixture. When the additions were completed the reaction mixture was stirred for a further 30 min. at −5°. The precipitate of NaCl and inorganic P was removed by filtration on a Büchner funnel (Whatman no. 531 paper) and the solid residue extracted with 200 ml. of ice-cold water in a Waring Blendor. The mixture was filtered and the residue re-extracted twice with 100 ml. of water. The residue was discarded; the four filtrates were combined and allowed to stand for 24 hr. at 5°. The crystalline precipitate which formed was removed by filtration and discarded, and the filtrate was adjusted to pH 7·5 by the addition of 5N-HCl. Ethanol (4 vol.) was then added and the oil which separated allowed to settle for 24 hr. at 5°. The supernatant liquid was discarded after removal by decantation and the semi-solid oil dissolved in 500 ml. of water. This solution was clarified by filtration and the sodium salts were again precipitated as an oil by the addition of ethanol (4 vol.). This mixture was allowed to stand for 24 hr. at 5°, the supernatant decanted and the oil dissolved in 500 ml. of water and filtered. These two precipitations removed the free taurocyamine and a large proportion of the inorganic P (Table 2).

Isolation of monophosphotaurocyamine. The aqueous solution of the twice-precipitated oil was

Table 2. *Analytical data on fractions obtained in the isolation of barium salts of monophosphotaurocyamine and fraction II*

Fraction	Vol. (ml.)	Taurocyamine (g.) Free	Total	Bound	Ba salt (g.)
Initial filtrate	1220	6·2	15·3	9·1	—
Soln. of oil (first ppt.)	500	0·3	8·3	8·0	—
Soln. of oil (second ppt.)	500	0·03	7·6	7·6	—
Fraction 0	960	0·03	0·7	0·7	0·9
Monophosphotaurocyamine	2980	0·01	4·7	4·7	10·8 (*7·0)
Fraction II	540	0·003	2·2	2·2	9·7 (*5·5)

* These figures represent the final yields after two further precipitations in the absence of added Ba acetate. This procedure results in appreciable losses but is necessary if serious contamination of the product with barium acetate is to be avoided.

passed through a column (66 cm. × 3 cm.) of De-Acidite FF (Cl^- form) at approx. 5 ml./min. and the effluent tested at intervals for the presence of bound taurocyamine. In all preparations it was found that some bound guanidine (fraction 0) passed through the column to the extent of 1–10% of the total bound guanidine present in the original solution. Chromatographic examination of this material after hydrolysis and development on paper in methanol–acetic acid–water (80:10:20, by vol.), *n*-butanol–6N-HCl (70:30, v/v) (Makisumi, 1952) and phenol–water (80:20, w/v) saturated with SO_2 (Crawford, 1951) indicated the presence of two guanidines. One of these was identical in behaviour with taurocyamine but the other, which had a higher R_F in all solvents, was not identified. This material was discarded. The column was then washed with water until the effluent was free of bound taurocyamine. Elution was then commenced with 0·2M-NaCl (5 ml./min.) and the eluate collected in 200 ml. fractions. Qualitative tests for bound taurocyamine were carried out on each fraction and fractions 1–15 were combined. Elution with additional 0·2M-NaCl removed only trace amounts of bound taurocyamine. Barium acetate (75 ml. of M-solution) was then added to the combined eluates and the pH adjusted to 8·2 by the addition of N-NaOH. The precipitate was removed by filtration and the filtrate adjusted to pH 4–5 by the addition of 5N-HCl. The solution was then placed in a flask, which was evacuated on a water-pump to remove CO_2, and the solution brought to pH 6–6·5 by the addition of a freshly prepared solution of NaOH. Ethanol (3 vol.) was added and the amorphous precipitate collected by centrifuging, washed with ethanol and ether and dried *in vacuo* over $CaCl_2$. The dry material was dissolved in 250 ml. of water, adjusted to pH 6 and precipitated by the addition of ethanol (2 vol.) in the absence of added barium acetate. The precipitate was collected as before, dried and again similarly precipitated. Yield, 7 g. (Table 2).

The material on analysis gave C, 8·8; H, 2·8; N, 9·6; S, 7·1; P, 7·3; Ba, 31·6; taurocyamine, 39·0; H_2O, 8·7; ash ($Ba_2P_2O_7$), 52·5. Calc. for $C_3H_8O_6N_3SPBa,2H_2O$: C, 8·6; H, 2·9; N, 10·0; S, 7·6; P, 7·4; Ba, 32·7; taurocyamine, 40·0; H_2O, 8·6; ash, 53·8%. The compound is believed to possess the structure:

$$HN=C(NHPO_3Ba)NH\cdot CH_2\cdot CH_2\cdot SO_3H,2H_2O.$$

Attempts to isolate the compound as the crystalline sodium, potassium and lithium salts were unsuccessful because of their markedly hygroscopic nature, but the crystalline ammonium salt was prepared. The barium salt (400 mg.) was dissolved in 20 ml. of water and passed through a column (1 cm. × 25 cm.) of Zeo-Karb 225 (NH_4^+ form). The column was washed with water and the washings (30 ml.) were combined with the original effluent. Ethanol (6 vol.) and acetone (6 vol.) were then added and the finely dispersed oil which separated crystallized in fine needles on standing at 5° overnight. The material, on analysis, corresponded to the empirical formula $C_3H_8O_6N_3SP(NH_4)_2$.

Isolation of fraction II. After elution of monophosphotaurocyamine the column was treated with 2M-NaCl to elute fraction II, which was collected in 540 ml. of eluate. Barium acetate (50 ml. of M-solution) was added and the solution became slightly opalescent but cleared when adjusted to pH 4–5 by the addition of 5N-HCl. Dissolved CO_2 was removed as before and the pH brought to 7. (At higher pH values a precipitate appears and this increases with increasing alkalinity.) Ethanol (2 vol.) was then added and the pH re-adjusted to 7 by the addition of 5N-NaOH. The precipitated barium salt was recovered by centrifuging, washed with ethanol and ether and dried *in vacuo* over $CaCl_2$. The dry material was dissolved in water and precipitated in the absence of additional barium acetate with ethanol (2 vol.). The precipitate, after drying, was redissolved, similarly reprecipitated and again dried. Yield, 5·5 g. (Table 2).

Fraction II gave on preliminary analysis a Ba:P:taurocyamine ratio of 2:2:1, and it was anticipated that the compound would be diphosphotaurocyamine and that its structural formula could be expressed thus:

$$BaPO_3\cdot N=C(NH\cdot PO_3Ba)NH\cdot(CH_2)_2\cdot SO_3H.$$

Full elementary analysis, however, revealed the presence of an excess of C, H and S over that demanded by theory on the basis of the above formulation. It seemed likely that the excess of these elements might be due to a contaminant eluted from the column by the 2M-NaCl. To test this supposition some of the material was dissolved and the solution passed through another column of De-Acidite FF (Cl^- form). The column was washed with water and then with 0·1M-NaCl until appreciable amounts of bound taurocyamine appeared. The remainder of fraction II was then eluted with 0·4M-NaCl and the product isolated as a barium salt in the manner described above. On analysis the material gave figures similar to those mentioned above. Attempts made to crystallize the compound as an ammonium, potassium or sodium salt were unsuccessful. The questions whether fraction II was a diphosphoguanidine and whether taurocyamine was in fact the guanidine released under mild conditions of hydrolysis were checked by the chromatographic behaviour of the guanidine released by hydrolysis in 0·1N-HCl at 65°; the material was chromatographed (Whatman no. 3MM) in a variety of solvent systems and in all

cases behaved similarly to authentic taurocyamine. The solvent systems used were acetone–aq. 2N-NH_3 soln.–water (60:30:10), propanol–aq. NH_3 soln. (sp.gr. 0·88)–water (60:30:10), Hanes & Isherwood (1949), and pyridine–*iso*pentanol–water (80:40:70), Roche, van Thoai & Hatt (1954).

Stability in acid. The relative stabilities of monophosphotaurocyamine and fraction II were determined by hydrolysis in N-HCl at 37°. With the monophosphate, P and taurocyamine were released in equimolar proportions throughout the course of the hydrolysis. The compound was 50% hydrolysed in about 70 min.

The behaviour of fraction II under similar conditions presented a marked contrast. Thus, owing to a difference in the stabilities of the phosphoryl linkages, the P:taurocyamine ratio in the early stages of the hydrolysis was about 4:1 and fell as hydrolysis proceeded. Fraction II was 50% hydrolysed in about 130 min., at which time the P:taurocyamine ratio was about 2·3:1.

Stability in alkali. No taurocyamine was released from either the monophosphate or fraction II when heated in N-NaOH for 60 min. at 37°.

Action of phosphatase. In an attempt to obtain some idea of the nature of the phosphoryl linkages in fraction II and to compare its behaviour with that of the monophosphate both were incubated with alkaline phosphatase. The results indicated that P and taurocyamine are released from the monophosphate in equimolar proportions throughout the course of the reaction and that the compound is completely hydrolysed by the enzyme. With fraction II (Fig. 1), on the other hand, the rate of liberation of P was much greater than that of taurocyamine. Thus at the end of 5 min. the molar ratio P:taurocyamine was 18 and after 20 min. had fallen to 5. After 75 min. both P and taurocyamine were being released at a slow constant rate with a P:taurocyamine ratio of approx. 2·6. Fraction II was hydrolysed only to the extent of about 55% after 240 min.

Taurocyamine phosphokinase experiments. The ability of fraction II to function as a substrate for the enzyme taurocyamine phosphokinase (TPK), which catalyses the reaction

phosphotaurocyamine + adenosine diphosphate ⇌ taurocyamine + adenosine triphosphate,

has been tested in the presence and absence of myosin. This latter enzyme was added to ensure that the maximum amount of taurocyamine would be released. Similar experiments carried out as controls, with the monophosphate as substrate, indicated (Table 3) that at equilibrium 55% of the taurocyamine was released. In the presence of myosin all bound taurocyamine was released. Such a result would, of course, be expected as the monophosphate is the natural substrate for the enzyme. The results with fraction II indicate that only a small proportion of bound taurocyamine is released even in the presence of myosin. It is of interest to note that the ratio of the taurocyamine present at equilibrium to that present when myosin was added is the same for both the monophosphate and fraction II.

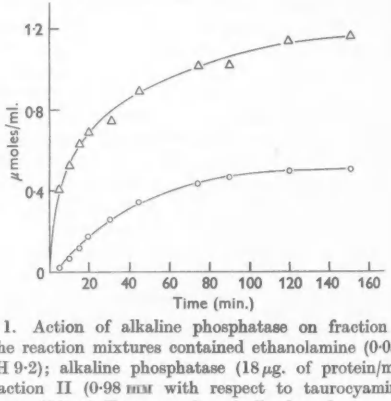

Fig. 1. Action of alkaline phosphatase on fraction II. The reaction mixtures contained ethanolamine (0·05M, pH 9·2); alkaline phosphatase (18 μg. of protein/ml.); fraction II (0·98 mM with respect to taurocyamine). Temp. 37°. ○, Taurocyamine; △, P released.

Table 3. *Monophosphotaurocyamine and fraction II as substrates for taurocyaminephosphokinase*

Reaction mixture: adenosine diphosphate (mM); Mg^{2+} ions (2·5 mM); Ca^{2+} ions (5 mM); *N*-ethylmorpholine (0·05M, pH 7·2); TPK (50 μg. of protein/ml.); monophosphotaurocyamine (2·67 mM) or fraction II (1·33 mM, calculated on bound taurocyamine content). Total volume, 1·5 ml. Reaction time, 8 hr. at 37°.

	Monophosphotaurocyamine		Fraction II	
	No myosin (*a*)	Myosin (800 μg. of protein/ml.) (*b*)	No myosin (*a*)	Myosin (800 μg. of protein/ml.) (*b*)
Taurocyamine (μmoles/ml.)	1·48	2·67	0·16	0·28
Taurocyamine (% released)	55	100	12	21
a/*b*	0·55		0·57	

DISCUSSION

The reaction between phosphoryl chloride and glycocyamine or creatine to yield *N*-phosphorylated derivatives was initially described by Zeile & Fawaz (1938) and subsequently by Ennor & Stocken (1948), who isolated phosphocreatine as the sodium salt. These communications did not refer to any side reactions and only one product was isolated. It was therefore anticipated that the phosphorylation of taurocyamine with phosphoryl chloride under similar conditions would yield but one product, i.e. a monophosphate. Such, however, was not the case and the separation of this latter compound from a mixture could be accomplished only by the use of an anion-exchange resin, from which it was eluted by 0·2 M-NaCl. Although the compound could be crystallized as the ammonium salt it was isolated as a pure amorphous barium salt.

Experiments with acid hydrolysis indicated the usual lability of the N–P bond, but it is of some interest to note that the compound is much more stable in acid than is phosphocreatine. Thus this latter substance is 50% hydrolysed in 0·1 N-HCl at 37° in 15 min. and is completely hydrolysed in 90 min., whereas monophosphotaurocyamine is 50% hydrolysed in N-HCl at 37° in 90 min. In the presence of acid molybdate phosphocreatine is more rapidly hydrolysed than in acid alone, but acid molybdate has no effect on the rate of hydrolysis of phosphotaurocyamine.

This lack of influence of molybdate on the hydrolysis of the monophosphate is of interest in view of the results of Baldwin & Yudkin (1950). These authors found that the rate of hydrolysis of the phosphagens of certain annelids and gephyreans in acid was retarded in the presence of molybdate. Moreover the 'retardation factor' was smaller than could be explained on the basis of the presence of phosphoarginine, the hydrolysis of which is markedly inhibited by molybdate. The monophosphate is completely hydrolysed by alkaline phosphatase, which is believed to attack only N–P and ester–phosphate linkages (Morton, 1955). In the dry form as the barium salt it is unstable and slowly breaks down over 6 months at room temperature to yield the free base and inorganic P. It has, however, been found that the compound is stable in solution as the sodium salt if stored at $-15°$; such solutions have been kept for 6 months without decomposition.

The other product of the phosphorylation is readily separated from the monophosphate but is of unknown constitution except insofar as it yields, on acid hydrolysis, 2 moles of P to 1 mole of taurocyamine. There is, however, no evidence as to the manner in which the P is bound, although it is clear from the phosphatase experiments that a part of the P is bound in an N–P linkage. If it be assumed that monophosphotaurocyamine is the natural substrate for TPK, and that the enzyme has a high degree of specificity for this substrate, additional evidence as to the nature of fraction II can be obtained from the action of this enzyme upon the monophosphate and fraction II. The fact that the ratio of the taurocyamine released in the absence and in the presence of myosin is about the same with monophosphotaurocyamine and fraction II as substrates is indicative of a contamination of this latter with the monophosphate. Thus it may be calculated that fraction II is contaminated to the extent of 20% with monophosphotaurocyamine. It is, however, possible that such a contamination arises as a result of breakdown of the original 'compound' as first isolated from the ion-exchange resin. This is perhaps likely, particularly as fraction II when isolated as the barium salt breaks down in such a way that 50% of the bound taurocyamine appears as the free guanidine even when stored in the dry state at room temperature (24°) over 4 months.

Just before submission of this paper for publication the report of the details of the synthesis and isolation of the ammonium salt of phosphotaurocyamine by van Thoai & van Thiem (1957*b*) came to hand. Phosphorylation of taurocyamine, as in the present work, was carried out with phosphoryl chloride but under different conditions, and there was no evidence for the formation of a phosphorylated product other than monophosphotaurocyamine. The isolation procedure involved the handling of large volumes and is thus less convenient than the method described here.

SUMMARY

1. A method is described for the preparation of the barium salt of *N*-phosphoguanidinoethanesulphonic acid.

2. A phosphorylated product in addition to the above is obtained. This can be readily separated from the reaction mixture but is of unknown constitution.

3. Some of the properties of *N*-phosphoguanidinoethanesulphonic acid are described.

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Biosynthesis of Intestinal Mucins

2. INCORPORATION OF [^{35}S]SULPHATE BY GUINEA-PIG COLON *IN VITRO**

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In a previous paper (Pasternak, Kent & Davies, 1958) the uptake of [^{35}S]sulphate by isolated gastrointestinal tissues of a number of animals was surveyed; the highest incorporation was found to occur in guinea-pig colon. This tissue was accordingly chosen for a more detailed investigation of fixation of [^{35}S]sulphate. A brief account of part of this work has already been presented (Pasternak, Kent & Davies, 1956).

METHODS

Preparation of tissue. Tissue was prepared as described by Pasternak *et al.* (1958).

Incubation. The incubation medium was that described by Pasternak *et al.* (1958), glucose being omitted. Substrates were added to yield final concentrations of 0·02 M, and incubated with tissue for 3 hr. except where otherwise stated; sodium pyruvate was kindly given by Mr R. W. Wakelin. Other details of incubation were as described by Pasternak *et al.* (1958).

Extraction of tissues. Tissues after incubation were extracted with 5 ml. of NaOH (2%, w/v) for 12 hr. at room temperature. The resulting extracts were centrifuged to remove residual cell fragments before dialysis. This treatment did not result in significant loss of bound sulphate by hydrolysis.

Dialysis. Dialysis of extruded mucin (i.e. that which passes into the incubation medium) and extracted mucin (i.e. mucin obtained from the tissue after incubation) (as defined by Pasternak *et al.* 1958) was carried out in cellophan against running tap-water (16°), constant activity being reached within 90 hr. Carrier Na_2SO_4 and penicillin plus streptomycin were added every 12 hr. as described by Pasternak *et al.* (1958).

Electrophoresis. The method of Kunkel & Tiselius (1951) was employed, sodium diethylbarbiturate buffer (0·07 M, pH 8·8) and Whatman no. 1 paper (8 cm. × 32 cm.) being used. Three mucin samples (0·025–0·1 ml.) were investigated simultaneously; by using three paper strips and four glass plates 'sandwich-wise' up to nine samples could be included in one run. At the end of electrophoresis (6 hr., 120 v; 4·3 v/cm.) the paper was dried and cut into longitudinal strips, each strip containing the separated components of one sample.

Dry-weight determination. Dry weights of tissues were determined as described by Pasternak *et al.* (1958).

Radioactive assay. Samples of the mucin solutions before and after dialysis were assayed as described by Pasternak *et al.* (1958). Alternatively, the proportion of added isotope in the [^{35}S]mucin was obtained by paper electrophoresis of a sample (0·05–0·08 ml.) of the medium or tissue extract after incubations.

Paper strips after electrophoresis were assayed (GM mica end-window tube EHM 2s operating at 1640 v) as contiguous rectangles (1·7 cm. × 1 cm.) along the length of the strip. The radioactive peak at the origin was identified as [^{35}S]mucin, whereas that which had migrated towards the anode was shown to be free sulphate (approx. $1{\cdot}4 \times 10^{-2}$ cm./v/hr.). The term [^{35}S]mucin is used throughout this paper to denote ^{35}S-labelled, [^{35}S]sulphate-free extruded or extracted mucin. It was considered unnecessary to correct for self-absorption, since the thickness of the paper, which is decisive for this isotope, is the same for both radioactive components. The radioactivities of the two components were determined by summing the counts below each of the plotted peaks and correcting for background (approx. 10 counts/min./cm.). The proportion of added isotope found in the [^{35}S]mucin was calculated from the ratio {[^{35}S]mucin (counts/min.) × 10^5}:{[^{35}S]sulphate + [^{35}S]mucin (counts/min.)} and expressed as counts/min./100 000 counts/min. initially added/mg. of tissue dry wt. (subsequently abbreviated as counts/mg. of tissue). [^{35}S]Mucin (counts/min.) represents the total counts given

* Part 1: Pasternak, Kent & Davies (1958).

by a mucin band on an electrophoretic strip, and [^{35}S]-sulphate the counts of the sulphate band on the same strip.

Amino sugar content and penetration by [^{35}S]*sulphate.* These were determined as described by Pasternak *et al.* (1958).

RESULTS

Determination of [^{35}S]*mucin.* In the early part of this investigation the amount of [^{35}S]sulphate incorporated during incubation was determined on measured samples of mucin which had been dialysed against distilled water for 90–130 hr. (Pasternak *et al.* 1958) or against running tap-water for 90 hr. The dialysis procedure, however, is not completely satisfactory; Bostrom & Mansson (1953) were unable to remove free sulphate completely from extracts of cartilage by dialysis, and therefore used a method of separation on Dowex 2 columns. We have not encountered this difficulty (see Table 2). On the other hand, another disadvantage of the dialysis procedure is the resulting change in volume of mucin solution. Such volume changes make calculation of total [^{35}S]mucin difficult. We have therefore employed paper electrophoresis between glass plates for the separation of free sulphate and incorporated sulphate. The advantage of this method as an analytical tool is that only a small portion of mucin solution (<0·1 ml.) is required and that several determinations can be made within a day (cf. 90 hr. for complete dialysis). On the other hand, the counting efficiency in assaying [^{35}S]mucin or [^{35}S]sulphate on paper (electrophoretic procedure) is only 25% of that on Perspex disks (dialysis procedure).

In order to isolate [^{35}S]mucin remaining after a portion of solution had been used for electrophoretic assay, dialysis, as described above, followed by freeze-drying, was employed.

A comparison of the results of dialysis and electrophoresis in determining incorporated [^{35}S]-sulphate is given in Table 1. The agreement in percentage counts incorporated (from which counts/mg. of tissue are calculated) indicates that non-dialysable ^{35}S may be equated with electrophoretically immobile ^{35}S, and provides evidence for the reality of the incorporation process.

Values of incorporated ^{35}S quoted subsequently were obtained either by dialysis (provided that large volume changes did not occur) or by electrophoresis. Each experiment contained an internal control (tissue incubated for 3 hr. without addition of substrate). The values of sulphate incorporation in Table 3 are calculated as ratios of this control.

Hydrolysis of [^{35}S]*mucin.* A sample of ^{35}S-labelled mucin was hydrolysed (2 hr., 4N-HCl at 110°), and the solution was evaporated *in vacuo* over KOH and examined by paper chromatography (M-ammonium acetate–ethanol, 2:5, v/v) on Whatman no 1 paper. Included on the chromatogram were samples of unhydrolysed mucin and the following amino acids: methionine, cystine, taurine and cysteic acid. Fig. 1 shows that at least 90%

Table 1. *Determination of* [^{35}S]*mucin by dialysis and electrophoresis*

Values (in counts/min.) refer to the extruded mucin fraction obtained during a typical experiment. Experimental details and corrections are described under Methods. Incorporation is the percentage of the added counts in the mucin after incubation. Calculated from paper-electrophoretic separations.

Dialysis		
Non-dialysable counts (0·2 ml.)	Total counts (0·05 ml.)	Incorporation (%)
4656	43 680	2·7
Electrophoresis		
Immobile counts (0·1 ml.)	Total counts (0·1 ml.)	Incorporation (%)
501	18 300	2·7

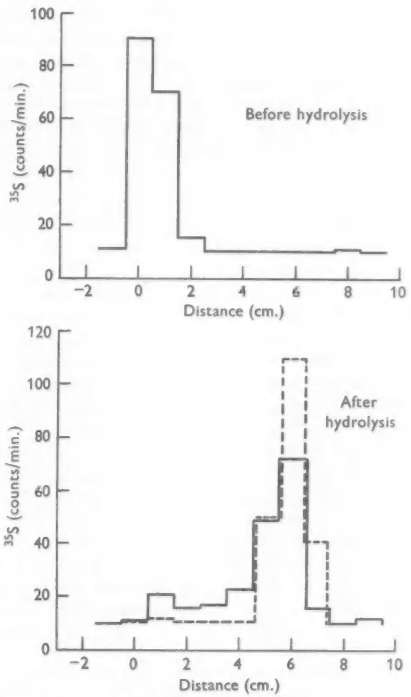

Fig. 1. Chromatographic analysis (ethanol–ammonium acetate) of [^{35}S]mucin (continuous line) before and after hydrolysis with 4N-HCl at 110° for 2 hr. [^{35}S]Sulphate marker is shown as a broken line.

of the original radioactivity can be recovered as free sulphate. The R_F of sulphate in this system is 0·23, and that of the other compounds is: cysteic acid 0·26, cystine 0·36, taurine 0·60 and methionine 0·70.

Effect of time on incorporation of [^{35}S]*sulphate.* In order to examine the fixation of [^{35}S]sulphate more closely, segments of colon were incubated for varying periods of time. Fig. 2 shows the result of such an incubation. The rate of appearance of [^{35}S]mucin fractions begins to fall in both fractions after 1 hr.; this decrease is accompanied by a decrease in oxygen uptake. (Reduction of Q_{O_2} is less marked in the presence of glucose.) Decrease in rate of uptake of [^{35}S]sulphate with time was also observed in an experiment in which tissues were pre-incubated in the absence of [^{35}S]sulphate for varying periods up to 2 hr. At the end of these times [^{35}S]sulphate was added and tissues were incubated for a further 3 hr. Tissues which had been pre-incubated longest fixed the least [^{35}S]sulphate.

Effect of various treatments. The results are summarized in Table 2; incorporation of [^{35}S]-sulphate by extracted mucin was similar to that by the extruded fraction. Incubation in nitrogen abolished uptake of [^{35}S]sulphate almost completely. This suggests that the incorporation process is an endergonic one, a view supported by the effect of certain inhibitors (Table 3). Heating the tissue to 100° or freezing it in liquid air before incubation abolished uptake of [^{35}S]sulphate completely.

The fourth treatment cited in Table 2 consisted of incubating tissue without [^{35}S]sulphate for 2 hr. The tissue was then removed and [^{35}S]sulphate added to the medium, which now contained extruded mucin; this was re-incubated for a further 3 hr. No radioactivity was found in the extruded fraction at the end of dialysis. This experiment shows that preformed mucin in the absence of tissue does not incorporate [^{35}S]sulphate; moreover, it provides a useful control for the dialysis procedure, since it demonstrates that free [^{35}S]sulphate is completely removed in the presence of unlabelled mucin. Lack of uptake of ^{35}S without tissue suggests that the necessary enzymes are intracellular and are not extruded with mucin.

A further experiment indicates that sulphatases are not present in extruded mucin; tissue was incubated in medium containing [^{35}S]mucin instead of [^{35}S]sulphate: no dialysable ^{35}S-labelled products were formed, nor was there any penetration of tissue by [^{35}S]mucin; reabsorption of mucin (albeit a dialysed and freeze-dried product) does not therefore appear to occur *in vitro*. The behaviour of homogenized tissue towards [^{35}S]sulphate has also been studied and will be discussed later.

Effect of added substrates. The effect of added substrates on oxygen uptake, penetration of [^{35}S]-sulphate and incorporation of [^{35}S]sulphate by extruded mucin is shown in Table 3; similar results were obtained with extracted mucin. The substrates which cause significant stimulation are D-glucose (Q_{O_2}, penetration by [^{35}S]sulphate and incorporation of [^{35}S]sulphate), sodium pyruvate (Q_{O_2} and [^{35}S]sulphate incorporation) and D-glucosamine ([^{35}S]sulphate incorporation).

Sodium fluoroacetate, phenol and salicylic acid (all at 20 mM) are inhibitory. Since these substances

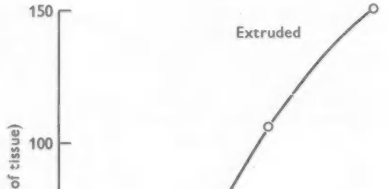

Fig. 2. Incorporation of [^{35}S]sulphate into extruded (○) and extracted (●) mucin factions by sections of guinea-pig colon incubated at 37° in Krebs–Ringer buffer.

Table 2. *Effect of various treatments on* Q_{O_2} *and incorporation of* [^{35}S]*sulphate*

Some values are averages derived from several experiments, the number performed being given in parentheses. Experimental details are described under Methods and Results.

Treatment	Q_{O_2} (at 150 min.)	Incorporation of [^{35}S]sulphate into extruded mucin fraction (counts/100 000 added/mg. of dry tissue after incubation for 180 min.)
Control	−4·6 (6)	14·1 (6)
Incubation in N_2	—	0·5 (3)
Heating tissue	0·0 (1)	0·0 (1)
Freezing tissue	−1·4 (1)	0·0 (1)
Removal of tissue from medium	0·0 (1)	0·0 (1)
Incubation with [^{35}S]mucin	−5·2 (4)	[a] (4)

[a] No [^{35}S]sulphate in extruded fraction; no [^{35}S]sulphate or [^{35}S]mucin in extracted fraction.

Table 3. *Effect of various substrates on Q_{O_2} penetration by [^{35}S]sulphate and incorporation of [^{35}S]sulphate*

Relative Q_{O_2} values, penetration by ^{35}S and incorporation of ^{35}S are calculated from the ratio: value with substrate/value without substrate. Most values are averages derived from several experiments, the number performed being given in parentheses. Experimental details are described in Methods. (In a preliminary communication (Pasternak, *et al.* 1956) glucosamine was stated to be without effect on uptake of [^{35}S]sulphate; subsequent electrophoretic measurements have shown this to be incorrect.)

Substrate	Relative Q_{O_2} (at 150 min.)	Relative penetration by ^{35}S (after incubation for 180 min.)	Relative incorporation of ^{35}S into extruded mucin fraction (after incubation for 180 min.)
D-Glucose	1·5 (11)	1·4 (3)	4·0 (9)
D-Glucosamine	1·0 (5)	1·0 (2)	1·8 (5)
D-Galactosamine	1·1 (3)	1·0 (2)	0·7 (3)
N-Acetyl-D-glucosamine	0·9 (3)	1·0 (2)	1·0 (3)
Sodium pyruvate	1·6 (5)	1·0 (3)	1·5 (5)
Sodium α-oxoglutarate	0·9 (4)	1·0 (1)	0·7 (4)
L-Glutamine	0·7 (2)	1·0 (2)	0·6 (2)
Potassium ethyl sulphate	0·9 (1)	—	1·0 (1)
Potassium methyl sulphonate	1·3 (1)	—	1·2 (2)
Sodium fluoroacetate (20 mM)	0·5 (1)	—	0·3 (1)
Phenol (20 mM)	0·2 (1)	—	0·0 (1)
Salicylic acid (20 mM)	0·0 (1)	—	0·0 (1)
Salicylic acid (0·5–5 mM)	1·0 (3)	—	0·8 (3)
2:4-Dinitrophenol (0·01–0·1 mM)	0·7 (6)	—	0·6–0·7 (6)

Table 4. *Amino sugar content of mucin fractions*

All values (μg./mg. of dry tissue) are averages of duplicate determinations. Experimental details are described in Methods and Results.

	Extruded mucin (during 30 min. preincubation)	Extruded mucin (during incubation for 120 min.)	Extracted mucin (after incubation for 150 min.)	Total mucin
Control	4·3 (2)	3·4 (2)	7·3 (2)	15·0
With glucose	4·3 (2)	3·7 (2)	7·2 (2)	15·2

abolish respiration almost completely it seems likely that their effect on uptake of [^{35}S]sulphate is due to a suppression of the energy supply (cf. Table 2). Lower concentrations of salicylic acid (0·5–5 mM) are without effect on incorporation of [^{35}S]sulphate, in contrast with the findings of Bostrom & Mansson (1953) and Loewi & Kent (1957); stimulation of respiration by these concentrations of salicylic acid was not observed (cf. the 'uncoupling' action of salicylic acid reported by Smith & Jeffrey, 1956).

Inhibition by low concentrations of 2:4-dinitrophenol (0·01–0·1 mM) is not very marked, and was not obtained without simultaneous depression of Q_{O_2}; 'uncoupling' of oxygen consumption and uptake of [^{35}S]sulphate by this substance has been shown by Boyd & Neumann (1954) and by Kodicek & Loewi (1955).

Sodium α-oxoglutarate, in contrast with sodium pyruvate, does not stimulate but rather depresses Q_{O_2} and incorporation of [^{35}S]sulphate; such an effect was also observed by Loewi (personal communication), working with guinea-pig granulation tissue.

The total amino sugar, which may be taken as a measure of total mucin, is unaffected by addition of glucose (Table 4). In this experiment tissues were incubated for 30 min. before being transferred to fresh vessels, in order to remove as much preformed mucin as possible (Pasternak *et al.* 1958). No stimulation of mucin production was apparent in any of the fractions. The time course of mucin extrusion during this experiment is shown in Fig. 3; the initial sharp rise represents extrusion of preformed mucin, which remains in spite of preincubation. Although the values in Fig. 3 are suggestive of an increase in total amino sugar during incubation, determinations could not in fact be made with sufficient accuracy to warrant such a conclusion. It is therefore not possible to say whether formation of [^{35}S]mucin represents a net increase of mucin, or whether there is an equivalent breakdown of unlabelled mucin during incubation.

DISCUSSION

The evidence which has been presented indicates that the mechanism by which [^{35}S]sulphate becomes incorporated into mucin of guinea-pig

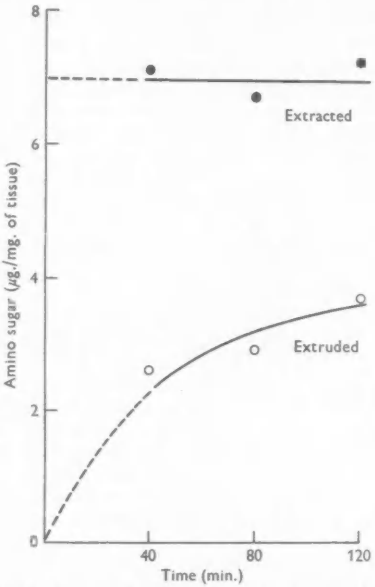

Fig. 3. Total amino sugar derivatives in the extruded (○) (i.e. soluble) and extracted (●) mucin fractions, formed during incubation of guinea-pig colon. Conditions of incubation and hydrolysis are given in Methods.

colon is one involving the formation of covalent ester linkages, rather than mere ionic exchange: thus [^{35}S]mucin does not lose [^{35}S]sulphate on dialysis or electrophoresis, nor is [^{35}S]sulphate fixed by mucin during incubation without tissue (Table 2). Furthermore, the incorporation process appears to be an enzymic one, requiring energy, since it is suppressed by heating or freezing the tissue (Table 2), by anaerobic conditions (Table 2), or by addition of inhibitors such as fluoroacetate (Table 3). The fact that incorporation follows a definite time course (Fig. 2) supports this view. Finally, it has been shown that the isotope is indeed present as bound [^{35}S]sulphate and that amino acids containing sulphur do not become labelled during incorporation (Fig. 1); this is in agreement with the findings of Kent, Whitehouse, Jennings & Florey (1956), that injected [^{35}S]-sulphate does not give rise to [^{35}S]amino acids in duodenal mucin obtained *in vivo*.

Such results as these have been obtained by workers studying incorporation of [^{35}S]sulphate by slices of cartilaginous tissue (Bostrom & Mansson, 1953; Kodicek & Loewi, 1955), and one may therefore conclude that certain aspects of the biosynthesis of intestinal mucin and chondroitin sulphate (formed in cartilage) are similar.

The present techniques enable closer study of the incorporation of [^{35}S]sulphate than hitherto; the overall process may be divided into three stages: (i) penetration of tissue by [^{35}S]sulphate; (ii) synthesis of [^{35}S]mucin; (iii) extrusion of [^{35}S]mucin. Penetration of tissue by [^{35}S]sulphate (stage i) is a rapid process, exchange being complete within 30 min. or less; this stage is stimulated by glucose (Table 3). Synthesis of [^{35}S]mucin (stage ii) and its extrusion (stage iii) have been followed over various time intervals during incubation (Figs. 2 and 3). It appears that extrusion is a faster process than synthesis, though the evidence is not conclusive. Extrusion is unaffected by glucose (Table 4), whereas synthesis is stimulated by glucose, glucosamine and pyruvate (Table 3).

Overall stimulation by glucose has been reported by several authors studying incorporation of [^{35}S]sulphate in cartilaginous tissue (Bostrom & Jorpes, 1954; Boyd & Neumann, 1954; Loewi & Kent, 1957). The extent to which synthesis of [^{35}S]mucin (stage ii) is so increased may be calculated in terms of [^{35}S]sulphate incorporated and compared with the amount of [^{35}S]sulphate which has penetrated the tissue (stage i). An increase of $1{\cdot}62 \times 10^{-2}$ μmole/mg. of dry tissue was found for incorporated sulphate in the presence of glucose, whereas the increase of free sulphate within the tissue was only $0{\cdot}085 \times 10^{-2}$ μmole/mg. of dry tissue. Stimulation of production of [^{35}S]mucin can therefore be only partly explained in terms of increased absorption of [^{35}S]sulphate by the tissue. It may be mentioned that the total amount of [^{35}S]sulphate incorporated in the presence of glucose is of the same order as that of free [^{35}S]sulphate in the tissue, indicating an appreciable turnover of the tissue-sulphate pool.

Stimulation of uptake of [^{35}S]sulphate by glucose is accompanied by an increase in Q_{O_2} (Table 3); Boyd & Neumann (1954), on the other hand, working with rat cartilage, reported a suppression of Q_{O_2}, whereas Loewi & Kent (1957) could detect no change in the respiration of guinea-pig granulation tissue in the presence of glucose. The effect of pyruvate (Table 3) is similar to that of glucose, causing stimulation of uptake of ^{35}S and Q_{O_2}; this suggests that the action of pyruvate, as of glucose, is concerned with supplementing the amount of energy available for synthetic processes (cf. Loewi & Kent, 1957). Stimulation of incorporation of ^{35}S by pyruvate, however, is not as marked as that by glucose. Glucose may therefore be affecting formation of [^{35}S]mucin by an additional mechanism, such as the formation of one of the precursors of [^{35}S]mucin; since galactose and glucosamine, both of which are constituents of intestinal mucin (Satoh, 1949; Werner, 1953; Whitehouse,

1955), are known to be formed from glucose under certain conditions (Leloir, 1955), this effect seems a likely one. It is supported by the observation that glucosamine itself is able to stimulate incorporation of [^{35}S]sulphate without altering Q_{O_2} (Table 3). This result is in accord with the finding of Bostrom, Roden & Vestermark (1955) and of Clark & Umbreit (1954), that glutamine is a powerful stimulant of incorporation of [^{35}S]sulphate by cartilage slices, since glutamine has been shown to be a possible amino-group donor in the glucose–glucosamine transformation (Pogell, 1956). We did not observe a glutamine effect (cf. Loewi & Kent, 1957); this discrepancy may be due to insufficient amounts of amino-group acceptors (such as glucose) within the tissue, or perhaps to a high level of glutamine in the tissue.

To summarize, the effects of glucose in stimulating synthesis of [^{35}S]mucin are: (*a*) increased penetration of tissue by sulphate; (*b*) additional energy is available for synthetic processes (cf. the action of pyruvate); (*c*) precursors of [^{35}S]mucin are formed and incorporated (cf. the action of glucosamine).

We think that the evidence which has been presented is sufficient to show that isolated segments of guinea-pig colon provide a useful system for the study of the biosynthesis of intestinal mucins. Subsequent investigations will be concerned with attempts to prepare active homogenates from this tissue and to isolate sulphate-containing intermediates.

SUMMARY

1. The mechanism of incorporation of [^{35}S]-sulphate by guinea-pig colon *in vitro* has been studied and found to be an enzymic process requiring energy.

2. Incorporated ^{35}S has been shown to be present as ester [^{35}S]sulphate in colonic mucin.

3. The effect of added substrates on respiration, penetration by [^{35}S]sulphate, incorporation of [^{35}S]-sulphate and total amino sugar content has been measured.

4. Sodium fluoroacetate, 2:4-dinitrophenol, phenol and salicylic acid inhibit respiration and incorporation of [^{35}S]sulphate.

5. Glucose stimulates respiration, penetration by [^{35}S]sulphate and incorporation of [^{35}S]sulphate; sodium pyruvate stimulates respiration and incorporation of [^{35}S]sulphate; glucosamine stimulates incorporation of [^{35}S]sulphate.

6. It has not been possible to decide whether the incorporation of [^{35}S]sulphate represents a net increase of mucin, or whether it is accompanied by an equivalent breakdown of preformed material.

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Chemical Modification of the Side Chains of Gelatin

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The reversible gel-forming power of gelatin has been the subject of numerous investigations, without the establishment of any certain knowledge of the molecular linkages involved. If the side chains of gelatin play any important part in the gel-forming property, their chemical modification should affect the latter. The hydrocarbon side chains are susceptible to reaction only under conditions that would cause very severe degradation of the gelatin molecule. They are, however, of only minor interest since they are unlikely sources of cross-linking in the gel. The polar groups present in the side chains are much more reactive, and the present paper describes their modification and the examination of consequent changes in physical properties. For convenience, these groups may be listed as in Table 1.

The carboxyl groups in gelatin (pK 3–5, Cohn & Edsall, 1943) are, with the exception of the phenolic hydroxyl groups (pK 10, Cohn & Edsall, 1943) originating from the traces of tyrosine, the only ones which produce a negative charge on dissociation, and are present in larger amount than any other ionizable group. They were converted into non-ionizable esters by reaction with methanol and an acid catalyst (Fraenkel-Conrat & Olcott, 1945).

Acetylation of gelatin with acetic anhydride in the presence of aqueous sodium acetate was selected as a convenient method of preventing the free amino groups from ionizing. Olcott & Fraenkel-Conrat (1947) claimed that this method, attributed to N. L. Hughes by Herriott (1947), resulted in acetylation of only the amino and, to a less extent, the phenolic groups of proteins. In non-aqueous media, acetic anhydride has been used for acetylation of stable proteins such as insulin (Herriott, 1947); Green, Ang & Lam (1953) have studied such a reaction on collagen; an investigation of the reaction is described here with gelatin under their conditions.

The guanidino groups of the arginine residues in gelatin are responsible for the existence of positive charges on the molecule at all readily measurable pH values, since these groups have a pK assumed to be in the region of 14 (Cohn & Edsall, 1943). The only reaction proposed as specific for the guanidino groups in proteins is that with hypobromite ion in strong alkali (Grabar & Morel, 1950). It was considered that the conditions of reaction would bring about other changes masking those that might be caused by de-guanidation, so the alternative approach of increasing the guanidino content was first investigated. The method adopted

Table 1. *Reactive groups in gelatin*

Quantities are in m-moles/g. of anhydrous ash-free gelatin, all determinations being made on the sample of gelatin used for the work described here.

α-Amino		$0{\cdot}01_5$	Courts (1954): end-groups assay with fluorodinitrobenzene
ε-Amino		0·43	Eastoe (1955)
		0·42	Kenchington & Ward (1954): titration-curve analysis
Carboxyl*		1·26	Eastoe (1955)
		1·23	Kenchington & Ward (1954): titration-curve analysis
Guanidino		0·48	Eastoe (1955)
		0·48	Kenchington & Ward (1954): titration-curve analysis
Hydroxyl:			
Hydroxyproline	1·09	1·75	Eastoe (1955)
Other aliphatic	0·65		
Phenolic†	0·01		

* In an acid-processed gelatin, this figure will include the amide groups which do not ionize in the range pH 2–12 and which are virtually completely hydrolysed to carboxyl groups in alkali-processed gelatin.

† Confirmed by unpublished work by the method of Udenfriend & Cooper (1952) applied to hydrolysed and unhydrolysed gelatin.

was that used by Hughes, Saroff & Carney (1949), which consists in the reaction of *O*-methyl*iso*urea with amino groups, at pH 10–11 and 0°. The guanidated lysine residues differ from those of arginine in having an extra methylene group, and are referred to as homoarginine residues. In addition, hydroxyhomoarginine is produced from hydroxylysine.

Apart from acetylation in non-aqueous media, no reactions were found which were suitable for modification of the hydroxyl groups. Attempts at sulphation with sulphuric acid (Reitz, Ferrel, Fraenkel-Conrat & Olcott, 1946) led to incomplete reaction and severe degradation; no success attended the use of sulphur trioxide under similar and modified conditions.

Preliminary mention of the work described here has been made by Ward (1953, 1955); the material formed part of a doctoral thesis (Kenchington, 1954).

MATERIAL AND METHODS

The gelatin used was a high-grade commercial material obtained by the alkaline process from animal skins. It had the following properties: viscosity 8·7 centipoises ($6\frac{2}{3}$%; 40°); logarithmic viscosity number (L.V.N.) 60; rigidity at 10°, 66×10^3 dynes/cm.^{2} [5·7% (w/w) concentration of anhydrous gelatin]; isoionic point pH 4·91.

The titration curve of this material has previously been described (Kenchington & Ward, 1954).

Moisture content. This was determined from the loss of weight on drying the powdered gelatin in a shallow metal dish for 18 hr. in a ventilated oven maintained at 105°. Ash content was measured by incinerating 0·5–1 g. of gelatin in a platinum dish at 510° until the residue was no longer black (1–4 hr.).

Titration curves. These were determined by the method of Kenchington & Ward (1954).

Logarithmic viscosity numbers. Values were obtained as described by Saunders, Stainsby & Ward (1954), and the rigidity of set gels was kindly measured by P. R. Saunders (Saunders & Ward, 1953). The L.V.N. is defined by

$$\text{L.V.N.} = \frac{\ln \eta/\eta_0}{C},$$

where C is the concentration (g./ml.) of solution, η is the viscosity of the solution and η_0 the viscosity of the solvent (International Union of Pure and Applied Chemistry, 1952).

Molecular weights. Dr G. Stainsby kindly determined the values in Table 2 by measurement of light-scattering (Stainsby, 1956).

Esterification of carboxyl groups

Gelatin, ground to pass a 60-mesh sieve, was stirred into 0·002–0·036 N-solutions of conc. H_2SO_4 in commercial absolute methanol at room temperature: 100 ml. of solution was used/g. of gelatin. The mixture was shaken at intervals during the first 2 hr. to prevent caking of the gelatin, which became considerably swollen. After standing for 18 hr. or more, with occasional shaking, the methanolic solution was decanted from the solid, which was then washed twice with four times its own volume of methanol before being sucked dry on a sintered-glass filter (grade 3). It was then washed twice with ether and again dried, first on the filter and then in a desiccator at 0·1 mm. Hg pressure. As the ether was removed, the swollen granules shrank to a fine powder indistinguishable in appearance from the starting material. This powder, swollen in ten times its own volume of water, made the supernatant liquor of pH below 2, so the latter was treated with 5 N-NaOH soln., with stirring, until pH 6 was reached and maintained, sufficient time being allowed for acid to diffuse out from the gelatin. At no time was the pH allowed to become greater than 6. The swollen granules were melted at 40° to a clear liquid which was set to a gel at 4°. This gel, cut to pieces, was dried in a current of filtered air, which was heated to 30–35° towards the end of the drying. During the drying process, movement of water to the surface of the jelly, followed by evaporation, produced a bloom of salts, some of which could be removed by brushing.

The prepared derivative was on occasion freed from salts and low-molecular material by rinsing with water to remove the surface salts, followed by soaking in large volumes of water at 0–4°, while suspended in a muslin bag, to reduce the total salt content. The water was changed three times in 24 hr., after which the swollen pieces of jelly were drained and dried as before, and ground.

Deionization was effected by melting the swollen pieces of jelly at 40° and passing them through a bed of mixed ion-exchange resins (Amberlite IR-120 and IRA-400), as in the determination of the isoionic point (Janus, Kenchington & Ward, 1951).

Estimation of methoxyl groups. The method and apparatus were essentially similar to those of Belcher, Fildes & Nutter (1955).

Acetylation procedures

Acetylation in aqueous solution. Powdered gelatin (59 g., air dry; 50 g., anhydrous) was allowed to swell in 1 l. of water at 10–15° for 1 hr. before being dissolved by warming at 40°. Anhydrous sodium acetate (25–150 g.) was dissolved in the warm solution, which was then cooled to 25° before acetic anhydride (0·5–400 g.) was added to the vigorously stirred solution, either dropwise or in portions of up to 20 ml. regulated to ensure that the temperature did not rise above 35°. When all the reagent had been added, the mixture was stirred for 10–15 min., poured into shallow Pyrex dishes and left to gel at 4° for at least 2 hr. The gel was cut into pieces, placed in a 12 l. glass tank and washed in running tap-water (5–10°) for 48 hr., the supply of water being sufficiently vigorous to stir the contents of the tank. Mechanical stirring was found unsatisfactory, since it broke up the pieces of swollen gel, causing loss of material and greater difficulty in manipulation. The pieces of gel became swollen (approx. 2% gelatin concentration) and of ash content 2–4% on dry weight by this treatment. Further washing in distilled water (3×10 l.) over 12–24 hr. greatly reduced the ash content. The gel was separated, melted, deionized (500 ml. of mixed resin bed), set, cut up, dried and ground as previously described. Measurement of the pH of the deionized liquid gave the isoionic point.

Acetylation in the absence of water. Two methods were

used. In the first, finely ground air-dried gelatin (23·5 g.; 20 g., anhydrous) was mixed with acetic anhydride (67 g.) at room temperature and left for 7 days at 4°. The mixture was then diluted with dry ether (500 ml.) and filtered on a grade 2 sintered-glass filter, the residue being washed with ether (5 × 100 ml.) and sucked dry. It was then kept at 0·1 mm. Hg air pressure for 24 hr. over NaOH (flakes). Half of the resulting powder was dissolved in water and electrodialysed at 35° between cellophan membranes for 24 hr. before being recovered as before.

In the second method, gelatin (59 g.; 50 g., anhydrous), acetic anhydride (350 g.) and acetic acid (240 g.) were mixed in a stoppered flask and left at 4° for 3 days. The greatly swollen mass was broken up and diluted with 2 l. of ether, with vigorous stirring, and filtered, washed with ether and dried as above.

Acetyl estimation. Hydrolysis of the acetylated gelatin (up to 1 g. containing not more than 0·5 m-mole of acetyl) was effected by heating with 20 ml. of 5 N-H_2SO_4 in a sealed Pyrex test tube for 5 hr. at 100° in a water bath. The hydrolysate was steam-distilled and titrated potentiometrically to pH 7 with 0·02 N-KOH, standardized under similar conditions. The solution was stirred by a stream of nitrogen bubbles.

O-Acetyl groups were determined in two ways, based on observations by Synge (1939) and Wolfrom, Konigsberg & Soltzberg (1936) that *O*-acyl and *O*-aryl derivatives are unstable to mild alkaline hydrolysis. In the first method, equal weights of acetylated gelatin and crystalline borax were dissolved in 100 ml. of water at 40° and maintained at that temperature for 1 hr. The product was dialysed, deionized and dried, and its acetyl content determined. The difference between this and the original value gave the *O*-acetyl value. In the second method, by using an apparatus similar to that used for the titration-curve determination (Kenchington & Ward, 1954), a solution of 1–3 g. of acetylated gelatin in 100 ml. of water was maintained at 40° and adjusted to pH 7·00 before being brought to and maintained at pH 9–10 by measured additions of standard alkali. When no further drift occurred (0·5 hr.), the pH was readjusted to 7·00 with a measured volume of standard acid, and the product dialysed, deionized and dried for acetyl determination, the *O*-acetyl content being obtained by difference in total acetyl before and after treatment. The volume of alkali added after adjustment to pH 7·00, less the volume equivalent to the acid used finally in returning to pH 7·00, gave the alkali consumed during deacetylation.

Guanidation procedure

O-Methyl*iso*urea was prepared as a solution at 0° in water. Gelatin powder was then stirred in, the ratio volume of solution : volume of gelatin being 20 : 1.

The weight of reagent used was varied from 10 to 100 times the theoretical amount required for the amino groups present; there was thus always a sufficient excess to maintain the pH of the gelatin at an alkaline value.

Rapid swelling of the gelatin occurred as soon as it was stirred into the reagent solution, the greater part of which was absorbed. The mixture was left at 0–4° for 7–8 days before being neutralized, with continuous stirring, with 5 N-HCl. The slurry was melted at 40° to a uniform solution, set to a gel, washed, deionized and dried.

Total nitrogen was determined by the method of Chibnall, Rees & Williams (1943), as modified by Eastoe & Eastoe (1954). Amino acid chromatography was performed by the methods of Moore & Stein (1951) in collaboration with J. E. Eastoe (Eastoe & Kenchington, 1954).

Oxidation of gelatin with hypobromite

Three solutions, each containing air-dried gelatin (23 g.; 20 g., anhydrous) dissolved in 1200 ml. of water, were cooled to 22° and treated in one of the following ways.

The first solution (*A*) was vigorously stirred while an alkaline solution of potassium hypobromite was rapidly added (the latter solution was made by dissolving 6 g. of bromine in a solution, at 0°, of 23 g. of KOH in 200 ml. of water). After 150 sec., a mixture of 22 ml. of 33 % HCl and 9 ml. of acetic acid was added with stirring, giving approx. pH 4.

The second solution (*B*) was treated with KOH (18·8 g.) and KBr (9 g.) dissolved in 200 ml. of water at 0° for 150 sec., before the same acid mixture as was used for solution A was added.

The third solution (*C*) was treated with KBr (9 g.), KCl (17 g.), potassium acetate (12 g.) and acetic acid (2 g.), all dissolved in 200 ml. of water.

All three resulting solutions were thus of identical salt content, it being assumed that the hypobromite was all reduced to bromide. All three were concentrated by distillation at reduced pressure to a volume of 300 ml., when they were found to be at pH 7 ± 0·5. After determination of rigidity and viscosity, the whole of the first two solutions were separately dialysed against distilled water (two changes of 10 l.) at 4° for 24 hr. The products were dried in polythene dishes in a current of air, yielding 20 and 21 g. respectively. A portion of each was redissolved and deionized to measure the pI (pH at isoelectric point). Paper chromatography of a portion of a hydrolysate of each was performed, after the methods of Consden, Gordon & Martin (1944).

Attempts were made to use milder reaction conditions, by lowering the pH of reaction from 14 to 8 in a series of experiments, and also by working with 0·5–1 % gelatin solutions at 4°, in the hypobromite-oxidation stage.

RESULTS

Methylated gelatin

The methoxyl contents of gelatin methyl esters increased according to (*a*) the concentration of the acid in the methanol used for esterification and (*b*) the time of treatment with the reagent, until a maximum value of 1·27 m-moles/g. was achieved, the constant value of 0·03 m-mole/g. obtained for the untreated material being allowed for. Washing in water at 4° for 24 hr. reduced the methoxyl content of the most highly methylated gelatins by up to 20 %. Except with slightly methylated gelatins, deionization greatly reduced the methoxyl content, the maximum value after deionization being 0·4 m-mole/g. Only a few of the cooled deionized solutions showed the characteristic isoelectric turbidity of gelatin solutions. Table 2 illustrates these results. The L.V.N. of the methylated gelatin fell with increase either in time of

Table 2. *Esterification of gelatin carboxyl groups*

L.V.N. is the logarithmic viscosity number; M_w is the weight-average molecular weight.

Sulphuric acid (m-moles/ 500 ml. of ethanol/5 g. of anhydrous gelatin)	Time of reaction (hr.)	Methoxyl content (m-mole/g.)	L.V.N.	M_w	$10^3 \times$ Rigidity (dynes/cm.^{2}) (10°)*	Methoxyl content (m-mole/g.)	After deionization	
							pH of liquor†	L.V.N.
1·8	18	0·05	63	—	65	0·06	4·9	—
3·7	18	0·11	61	200 000	—	0·11	5·0	—
7·4	18	0·39	53	—	—	0·29	8·7	—
11·0	18	0·71	45	—	—			
18·4	18	0·81	39	80 000	—	0·26	9·7	—
1·8	66	0·19	58	—	—	0·13	5·4	—
3·7	66	0·45	50	—	57	0·26	8·9	—
7·4	66	0·77	38	—	41	0·39	10·0	29
11·0	66	0·97	34	—	23	0·42	10·0	23
18·4	66	1·00	24	—	—	0·39	10·0	24
1·8	196	0·25	34	—	—	0·19	5·8	—
3·7	196	0·81	26	—	—	0·32	9·7	—
7·4	196	1·10	29	—	—	0·39	9·9	22
11·0	196	1·27	26	55 000	—	0·39	10·0	23
18·4	196	1·27	16	—	—	0·42	10·0	20
0·0 (control)	—	0·3	60	220 000	66	0·0	4·91	64

* Concentration: 5·7 % (w/w) anhydrous gelatin.
† This will be pI only for gelatins of low methoxyl content.

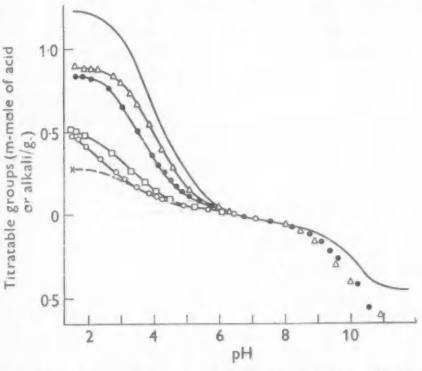

Fig. 1. Titration curves of gelatin methyl esters. Experimental points are not shown on the curve for the original gelatin (Kenchington & Ward, 1954). Gelatins of differing degree of esterification gave samples A (△), B (●), C (□), D (○). Point X is the corrected value for acid end-point of D; the broken curve from X indicates the probable true shape of the titration curve. Points on the alkaline side of A and B show the effect of alkaline hydrolysis; the positions of these points on the pH scale are a function of time of exposure to alkali. (See text for details.)

contact with the reagent or of catalyst concentration, corresponding to increase of methoxyl content. The deionized materials, however, of considerably reduced methoxyl content, showed no corresponding increases in L.V.N. as would have been expected were the original fall in L.V.N. attributable to esterification rather than degradation. The molecular weights confirmed that degradation had occurred.

The titration curves of the original and esterified gelatins are shown in Fig. 1, carboxyl titrations in the pH range 1·5–6·5 showing reductions corresponding to the degree of esterification. There were large variable increases in the titration at pH 8·5 and higher, illustrated by two series of points where considerable pH drifts occurred and the titration was non-reversible. With one gelatin, 'S', before and after acid titration (to pH 1·5) the material contained 0·44 m-mole of methoxyl/g., whereas after alkaline titration (to pH 11) the material recovered contained only 0·11 m-mole of methoxyl/g. In the latter, there was thus a loss of 0·33 m-mole of methoxyl/g.; the increase in free acid groups calculated from the non-reversibility of the titration was 0·2–0·4 m-mole/g. In the back-titration, acid exactly equivalent to the total alkali used in the titration was added. Any alteration in the number of ionizing groups titrated was at once indicated if the observed pH differed from the original pH; the amount of base required to produce this pH change could be measured, to give a quantitative measure of the change in the number of ionizing groups. Gelatins containing more than 0·8 m-mole of methoxyl/g. showed instability towards acid, illustrated by 'D' in Fig. 1, and gave a non-reversible titration on the

Table 3. *Comparison of analytical results for methoxyl*

The methoxyl content was determined by a modified Zeisel method (see Methods). The method of calculating the titration correction is described in the text.

Sample	Methoxyl content (m-mole/g.) After Zeisel	From titration curve
A	0·31	0·33
B	0·44	0·42
C	0·77	0·77
D	0·88	{0·76 uncorrected {0·9 corrected

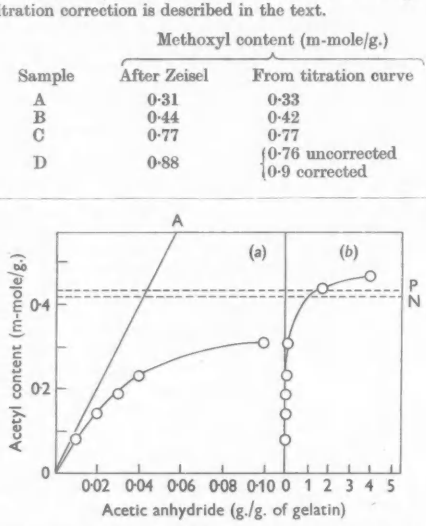

Fig. 2. Acetylation of gelatin, in aqueous sodium acetate solution, with acetic anhydride. Broken lines N and P show calculated contents of ε-acetamido and ε-acetamido plus α-acetamido of fully *N*-acetylated gelatin. (*a*) Line 0A is theoretical for 100% reaction of the anhydride with gelatin. Effect of small amounts of acetic anhydride. (*b*) Effect of larger amounts of acetic anhydride.

acid as well as the alkaline side, and a different shape in the titration curve on the acid side. When the end point of the titration curve on the acid side was corrected by deducting the titration due to additional carboxyl groups liberated by hydrolysis of ester groups (obtained as above from the non-reversibility), the anomalous form of the curve was explained. The methoxyl content calculated from the titration curve value after this correction was in good agreement with that obtained by the Zeisel estimation, as were the values for the other materials where no correction was necessary (Table 3).

Acetylation of gelatin

In aqueous sodium acetate solution, the degree of acetylation of gelatin depended on the amount of acetic anhydride added, as shown in Fig. 2 (*a*) and (*b*). A maximum acetyl value of 0·47 m-mole/g. was found. On treatment with alkali by either of the methods described, this was reduced to 0·43 m-mole/g. Acetylated gelatin of acetyl content not exceeding 0·43 m-mole/g. was stable to alkali under the conditions of the titration-curve determination, and showed a reduction in the titration of α- and ε-amino and histidine groups between pH 6·5 and pH 11·5, corresponding fairly well with the acetyl content (Fig. 3 and Table 4). The values calculated for amino substitution by the change in isoionic point, however, were not in good agreement, being low. The minimum isoionic point recorded was at pH 4·28. There was a small reduction in the carboxyl titration, of 0·06 m-mole/g.

The physical properties of the *N*-acetylated

Table 4. *Acetylation of gelatin in acetate buffer solution*

L.V.N. is the logarithmic viscosity number. For determination of acetyl content, see Methods. Acetyl content and titration values are expressed as m-moles/g. of anhydrous ash-free derivative.

Material	Acetic anhydride (g./g. of gelatin)	Acetyl content by chemical analysis	Titration values: pH 6·5–8·0	pH 8·0–11·5	Difference from original between pH 6·5 and 11·5	pI	Acetyl content calculated from pI*	L.V.N.	$10^3 \times$ Rigidity (dynes/cm.²) 0°	10°	20°
Original gelatin	0·0	0·0	0·06	0·42	0	4·91	0·0	60	101	66	26
U	1·7	0·44	0·05†	‡	‡	4·28	0·33	58	85	53	25
T	4·0	0·47	0·06†	‡	‡	4·29	0·33	46	75	45	13
T_a (after KOH)	—	0·42	—	—	—	4·39	0·31	—	—	—	—
T_b (after borate)	—	0·43	0·04	0·06	0·38	4·39	0·31	40	66	40	8
S	0·10	0·31	0·05	0·10	0·33	4·31	0·32	51	98	61	20
R	0·03	0·19	0·04	0·22	0·22	4·58	0·20	62	90	61	22
P	0·01	0·08	0·05	0·36	0·07	4·76	0·10	58	100	63	23
Q	0·02	0·14	0·05	0·28	0·15	4·65	0·13	56	98	61	22

* Allowance was made for decrease in carboxyl titration.
† May be high owing to the beginning of instability to alkali.
‡ No value was obtainable owing to pH drifts.

gelatin (Table 4) were very similar to those of the starting material.

The material produced by acetylation in acetic acid–acetic anhydride solution, of acetyl content corresponding to both *N*- and *O*-acetylation, swelled in water, but the swollen particles would not melt even at 100°. Similar behaviour was observed with a 50% (w/v) urea solution used as a hydrogen-bond breaker; after suspension for 2–3 hr. in a borax solution at pH 10, the swollen gel melted slowly at 30–40°, presumably owing to decomposition of alkali-labile intermolecular cross-links of ester type.

The material of low acetyl content produced by treatment with acetic anhydride alone was similar to the original gelatin in solubility, solution viscosity, and gel rigidity.

Guanidation of gelatin

The reaction products from *O*-methyl*iso*urea and gelatin were of increased nitrogen content, but unchanged pI. The titration curves differed from the original gelatin only in the range from pH 8 to 11·5, where the content of titratable groups was reduced as the nitrogen content increased, corresponding with increase in amount of the guanidating reagent used (Table 5). The viscosities showed a slight decrease, as did the rigidities. The most highly guanidated material, whose titration curve is given in Fig. 4, was analysed for basic amino acid content (Eastoe & Kenchington, 1954), and the chromatogram showed the emergence of two new peaks attributed to homoarginine and hydroxyhomoarginine. The analytical values are reproduced in Table 6.

Deguanidation of gelatin

The gelatin solution *A* which had been treated with alkaline hypobromite was yellowish brown and possessed an odour reminiscent of hypobromite, which was not lost on treatment with urea or sulphite solutions. The values for rigidity

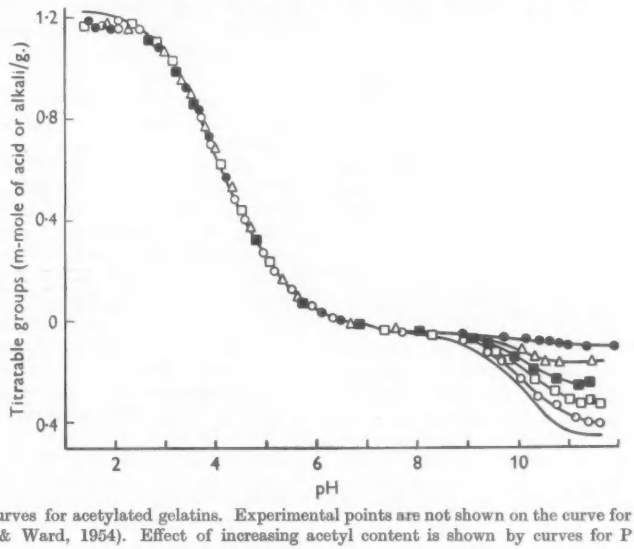

Fig. 3. Titration curves for acetylated gelatins. Experimental points are not shown on the curve for the original gelatin (Kenchington & Ward, 1954). Effect of increasing acetyl content is shown by curves for P (○), Q (□), R (■), S (△), T_b (●).

Table 5. *Analysis and physical properties of guanidated gelatin*

L.V.N. is the logarithmic viscosity number.

Material	% Total nitrogen	Decrease in titration pH range 8·0–11·5 (m-mole/g.)	L.V.N.	$10^3 \times$ Rigidity (dynes/cm.^{2}) 0°	10°	20°
Original	17·9	0	57	101	61	28
X	18·5	0·10	51	96	58	26
Y	18·5	0·33	51	97	55	27
Z	18·7	0·36	48	95	49	16

and viscosity are given in Table 7, and indicate severe degradation. The influence of the salts on the rigidity is seen to be large, as is the effect of alkali alone (solutions *B* and *C*). Similar results were obtained by treating 8% gelatin solutions at 40° with hypobromite at 0°.

Attempts were made to use milder conditions by reducing the pH of reaction and by working at 4° with dilute gelatin solutions to prevent gelation. It was found that at any pH below about 12, addition of hypobromite produced an immediate precipitate of what appeared to be cross-linked gelatin: physically this precipitate resembled that produced by organic solvents such as acetone or ethanol, but differed in that it appeared immediately on the addition of a small volume of reagent. It was insoluble in hot water but, if freshly prepared, could be redissolved in warm sulphurous acid solution.

When the reaction was carried out at pH 12–13, precipitates were formed initially but soon redissolved. Above pH 13 no precipitates were seen. Observations were impeded by the generation of large numbers of minute gas bubbles, and the necessity for high-speed stirring to ensure homogeneity in the short reaction time. The gas liberated was not investigated; it was assumed to be nitrogen (Chinard, 1948). Attempts to work at pH 8 or less brought about precipitation accompanied by liberation of bromine from the hypobromite under these less alkaline conditions.

The viscosity of solutions treated under these modified conditions, and which had not precipitated, was considerably reduced, as was the gelling power. The volume of ethanol required to bring about precipitation of the 'gelatin' from solution was increased by 200–300%; pI was variably reduced although the carboxyl titration appeared largely unaltered. Paper chromatograms showed that considerable amounts of arginine were still present in these materials.

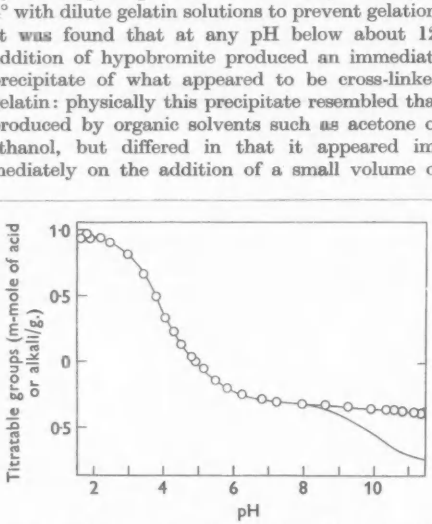

Fig. 4. Titration curves for original gelatin (—) and guanidated gelatin Z (○—○).

Table 6. *Amino acid content (m-moles/g.) of original and guanidated gelatin*

Residue	Material: Original		Material: Z
Lysine	0·312	0·34	0·01
Ornithine	0·027		
Hydroxylysine	0·077		0·003
Histidine	0·056		0·053
Arginine	0·476		0·532
Homoarginine*	0·0		0·280
Hydroxyhomoarginine	0·0		0·067

* Cf. Greenstein (1938).

DISCUSSION

Methylation of gelatin

The maximum methoxyl content achieved (1·27 m-moles/g.) agrees closely with the values of 1·23 m-moles/g. for free carboxyl in the gelatin used, obtained from the titration curve, and of 1·28 m-moles/g. from chromatographic analysis. Complete esterification of the carboxyl groups of gelatin can be considered to have been achieved. The blank of 0·03 m-mole/g. of 'methoxyl' is attributed to the thio-methyl group of methionine (Baernstein, 1932), compared with 0·05–0·06 m-mole/g. obtained by Eastoe (1955) using the method of Moore & Stein (1951).

The instability of the methyl esters to alkali is typical, and is responsible for the irreversible alkaline titration. It prevents any conclusion being drawn about the effect of the reaction on ε-amino groups, although the absence of any

Table 7. *Physical properties of hypobromite-treated gelatin*

Solution *A* was treated with hypobromite, solution *B* with the corresponding amount of alkali. Solutions *A*, *B* and *C* all had the same final concentrations of salts and protein. L.V.N. is the logarithmic viscosity number.

Solution	Untreated: L.V.N.	Untreated: $10^3 \times$ Rigidity (dynes/cm.^{2})	Deionized: L.V.N.	Deionized: $10^3 \times$ Rigidity (dynes/cm.^{2})
A	30	No gel	30	8
B	58	10	48	73
C	56	35	58	100

change in the α-amino plus histidine titration indicated little, if any, reaction of α-amino groups. The agreement between the Zeisel methoxyl value and that calculated from the titration curve is good.

The instability to acid, and even to water, of gelatin methyl esters appears to be confined to those of high methoxyl content, and may be due to the different carboxyl groups involved.

The hydrolysis on deionization is to be expected since the pI for gelatins of even moderate degree of methylation would be in the alkaline range, from pH 9 upwards, under which conditions ester hydrolysis is rapid, with a consequent fall in pI as carboxyl groups are freed. The loss of methoxyl groups would cease as the pI, and hence the pH, approached 7.

Physical measurements on the esterified gelatins indicated that severe degradation had taken place during their production. The fall in viscosity with time of reaction and amount of reagent, although corresponding to increased methoxyl content, appears due to increased hydrolysis. Thus there is no trace of any viscosity increase on reduction of methoxyl content by deionization. The rigidity of set gels of these materials is greatly reduced, which would be expected from the degradation indicated by the lowered viscosity and by reduced molecular weight. There is no evidence of a direct effect on the gelling power, i.e. on the 'rigidity factor' of Saunders & Ward (1955).

Acetylation of gelatin

The stability to alkali of the acetyl groups of acetylated gelatin, and the close relation of the maximum content of such groups to the free amino content of the original gelatin, indicate that the action of acetic anhydride on sodium acetate solutions of gelatin is to produce preferential acetylation of the amino groups. No other groups react until a large excess of reagent is used in order to complete the amino acetylation, when some acetylation of hydroxyl groups occurs. These *O*-acetyl groups are easily removed by virtue of their instability to mild alkaline hydrolysis.

The pH of the reaction was found to change very rapidly from an initial value near pH 8 to pH 4, as would be expected from the use of an acetate buffer. To maintain a reaction pH of 7–8, as claimed by Olcott & Fraenkel-Conrat (1947), it was necessary to add alkali continually. The specificity of the reaction under the conditions used cannot therefore be ascribed to hydrolysis of *O*-acetyl groups in a slightly alkaline medium, but must be due to the more rapid reaction of the amino groups.

Lea, Hannan & Rhodes (1951) acetylated casein by this method and found that neither the rate of addition of the anhydride nor the time of standing had any significant effect on the amino nitrogen; similar results have been obtained in this Laboratory with gelatin. The same authors considered that 94–97% acetylation of amino groups was achieved, but found a discrepancy of 20% more acetyl groups introduced than amino groups lost. Green *et al.* (1953) found it necessary to add alkali to maintain pH 8 when acetylating collagen by this method, and then obtained *N*-acetylation without *O*-acetylation; when no alkali was added some *O*-acetylation occurred.

The maximum of *N*-acetyl groups (0·43 m-mole/g.) reported here is in excellent agreement with the total amino content of 0·42–0·44 (Table 1), and with the maximum *N*-acetyl figure of 0·39 m-mole/g. of Green *et al.* when allowance is made for the absence of 0·02 m-mole/g. each of α-amino groups and ε-amino groups from ornithine. Attempts to measure the *O*-acetyl content by determination of the alkali neutralized during mild alkaline hydrolysis gave values (0·08–0·09 m-mole/g.) in disagreement with the results of total acetyl estimations on the purified material before and after the hydrolytic treatment (0·47–0·43 = 0·04 m-mole/g.).

Comparison of the acetyl values from chemical estimation with those from determinations of the titration curve and the isoionic point shows that there is agreement until the acetyl content rises above 0·3 m-mole/g., when discrepancies appear. The tyrosine content of gelatin should give a titration of 0·02 m-mole/g. from pH 8 to 11·5, whereas the minimum value obtained was 0·06 m-mole/g. A similar result was obtained with guanidated gelatin (cf. Figs. 3 and 4); no satisfactory explanation can yet be put forward. The titration curves of acetylated gelatins show a slight reduction in the carboxyl titrations (mean 0·06 m-mole/g.), which is of unknown origin and significance. The acetylations under anhydrous conditions support the findings of Green *et al.* (1953) that there is little acetylation unless acetic acid is added to the reaction mixture, when a high degree of *O*- and *N*-acetylation is achieved. These authors consider acetic acid to act as a catalyst; in view of the concentration required and of the fact that acetic acid has considerable swelling and solvent power for gelatin, whereas acetic anhydride has none and is soluble in acetic acid but not in water, it would seem probable that the function of the acetic acid is that of a solvent rather than a catalyst. The maximum acetyl content obtained by Green *et al.* for collagen was 1·75 m-moles/g.; with gelatin the same method gives 2·0–2·1 m-moles/g. The theoretical value for complete acetylation of amino groups, phenolic and other hydroxyl groups in gelatin is 2 m-moles/g., calculated from the amino acid composition found by Eastoe (1955). It is possible that the different values for collagen and

gelatin might be due to the presence of ester cross-links in collagen, as suggested by Gustavson (1955). The ability of highly acetylated gelatin to swell in water but not to dissolve on warming corresponds to the behaviour of cross-linked material. The solubility on treatment with dilute alkali suggests that cross-links of carboxylic ester type are involved, being readily hydrolysed by alkali. The use of anhydrides, including acetic anhydride, to promote condensation reactions has been reviewed by Tedder (1955), and there are in gelatin many carboxyl and hydroxyl groups which might undergo condensation.

It is possible that cross-linking reactions of this type may reduce the total acetyl content obtainable with collagen by immobilizing some of the reactive centres by internal cross-links, artificially introducing the type of bond suggested by Gustavson (1955) to occur naturally. The more ordered structure of collagen may facilitate such cross-linking reactions and render the blocking action more effective.

The fall in pI on acetylation is not as large as would be expected from the work of Hitchcock (1923) on deamination of gelatin, which gave pI 4 for completely deaminated gelatin. From the titration curve it can be calculated that since the removal of 0·4 m-mole of amino nitrogen/g. is equivalent to the addition of 0·4 m-mole of acid, the pI should move from pH 4·91 to 4·0, in agreement with Hitchcock's value. Since the conditions used by Hitchcock are known to cause reaction of groups other than amino, the position is far from clear. Deamination of the gelatin used in this work under mild conditions (20°, pH 4, 30 min.) gave gelatin having a pI of 4·26, the same as that of acetylated gelatin. The position is yet more complicated because Hitchcock (1923) used a different gelatin for his deamination work from the Eastman Kodak gelatin he used for his titration-curve determinations (1931). It was suggested from titration-curve analysis (Kenchington & Ward, 1954) that in the Eastman Kodak material a considerable amount of arginine had undergone conversion into ornithine, giving high amino nitrogen content. This suggestion has been confirmed by amino acid analyses of gelatin from the same source by Hamilton & Anderson (1954).

The effect of acetylation on the physical properties of gelatin is very small, there being a slight fall in viscosity and jelly strength with highly acetylated gelatins, attributable to slight degradation.

Guanidation of gelatin

The reaction of *O*-methyl*iso*urea with gelatin has been shown to be confined to the amino groups and to proceed to completion. There were indications that when an alkali such as potassium hydroxide was used to adjust the pH during reaction, instead of allowing excess of reagent in the free base form to maintain the pH, a lesser degree of reaction was achieved. The reaction with ornithine brings about its reversion to arginine, from which it is produced by the alkaline pretreatment of collagen in the manufacture of gelatin.

The results from total nitrogen, titration-curve and amino acid-composition determinations are all in fair agreement, and the discrepancies are only of the order of the limits of experimental error. Thus from chromatographic analysis the material titrating from pH 8 to 11·5 should not exceed 0·02 m-mole of ϵ-amino groups/g., plus 0·02 m-mole of phenolic groups/g., whereas the experimental value was 0·06 m-mole/g., as with acetylated gelatin. Theoretically, the nitrogen content should increase by a maximum value of 0·87 %; an increase of $0{\cdot}9 \pm 0{\cdot}05$ % was observed.

The values of certain physical properties (such as viscosity and gel rigidity) of the products show small decreases, quite in accord with a very small amount of degradation at pH 12–13 and 0°. There was certainly no sign of any increase in gel rigidity.

Deguanidation of gelatin

These experiments show that hypobromite as a specific reagent for arginine in the investigation of protein structures must be considered of limited use; any results based on it should be treated with reserve until confirmed by independent methods. Grabar & Morel (1950) attributed the gelling power of gelatin to guanidino groups, from results based on the use of hypobromite, supported by evidence which would more plausibly be interpreted as favouring a hydrogen-bonded structure for gelatin gels. The work described here shows that hypobromite not only degrades gelatin, but probably also cross-links it. The combination of both processes is closely akin to the controlled cross-linking with glyoxal, followed by degradation, used by Campbell *et al.* (1951) to reduce the ability of gelatin to gel while maintaining a high molecular weight. The original observation of Chinard (1948) was that hypobromite, while liberating nitrogen almost quantitatively from arginine in gelatin, attacked many other sites on the protein molecule.

General effects of side-chain substitution

None of the chemical modifications achieved has shown any significant effect on gel strength except that which could be attributed to the presence in the product of some material of reduced molecular weight, ineffective in forming the gel. For practical purposes, this degradation is indicated by change in viscosity. All the ionizable groups have, in turn,

been altered in some way in the course of the work, but not the unionizable hydroxyl groups which are present in large numbers and which may well play an important part.

It would seem that whatever effect the charged groups have is relatively small in this connexion; this correlates well with the slight dependence of gel strength on pH, except at extreme values.

Saunders & Ward (1955) and Pouradier, Venet & Trigny (1955) have shown that above a molecular weight of approximately 80 000 the rigidity is largely independent of molecular weight and of viscosity, whereas the rigidity of the gel decreases sharply as the molecular weight is reduced from this value. The existence of different gel-forming powers in proteins of the same molecular weight (the 'rigidity factor' of Saunders & Ward, 1955) may be explained if some considerable degree of order in the molecular structure is necessary to attain the maximum gel strength, and if the latter diminishes with loss of this ordered structure. The most probable linkage for joining these structure groups together is the hydrogen bond. There are vast numbers of sites suitable for its formation available in the molecule, including hydroxyl groups and peptide bonds, while the low heat of melting of the gel necessitates little change in energy. Also, all the known compounds which cause liquefaction of gels, without degradation, are active in hydrogen-bond breaking (Courts, 1957).

Subsequent to the completion of the work, a method for selective acetylation of hydroxyl groups in proteins has been published (Bello & Vinograd, 1956) which still, however, produces degradation. Bello (1956) has also introduced a new reagent, thionyl chloride, for esterification of gelatin, which does not appear to produce much degradation. His work was all performed on gelatin produced by an acid process. The results of physical measurements other than those of viscosity are not yet available.

SUMMARY

1. The ionizable groups in gelatin have been chemically modified in order to observe any resultant effects on the solution viscosity and gel rigidity. No effect not attributable to degradation was detected.

2. Complete esterification of carboxyl groups was achieved, since the analytical figures for methoxyl content agreed with the total free carboxyl content before treatment. Highly esterified material was unstable in aqueous solution at all pH values.

3. Complete *N*-acetylation was achieved by the action of acetic anhydride on gelatin dissolved in aqueous sodium acetate. Little *O*-acetylation occurred until the majority of the amino groups were acetylated. The *O*-acetyl groups were easily removed by mild alkaline treatment.

4. Guanidation of amino groups was shown to proceed virtually to completion.

5. Under all conditions of reaction studied, hypobromite was found to produce extensive degradation and other changes before its reaction with guanidino groups was complete.

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A Study of the Inhibition of Catalase by 3-Amino-1:2:4-Triazole

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Heim, Appleman & Pyfrom (1955, 1956) have shown that the injection of 3-amino-1:2:4-triazole into rats causes a rapid decrease of the catalase activity of the liver and kidney to very low levels, but has no effect on the catalatic activity of blood. These authors found that crystalline catalase was partly inhibited *in vitro* only at high concentrations of 3-amino-1:2:4-triazole (>0·05M); such concentrations were far above those necessary for the inhibition of catalase by 3-amino-1:2:4-triazole *in vivo*. The inhibition *in vitro* was reversible by dilution, whereas that obtained *in vivo* was not.

Sugimura (1956) showed that the incubation of rat-liver suspensions with 3-amino-1:2:4-triazole at 37° caused similar decreases in the catalatic activity of the suspensions, that the incubation of a purified crystalline preparation of ox-liver catalase with 3-amino-1:2:4-triazole did not result in any decrease of activity, but that when a liver extract was incubated with purified catalase in the presence of 3-amino-1:2:4-triazole the inhibition of catalatic activity occurred. In every case the inhibition required the presence of oxygen.

The present paper is concerned with the further investigation of these effects with simple systems *in vitro* and with the properties of the catalase inhibited by 3-amino-1:2:4-triazole.

MATERIALS AND METHODS

Catalase preparations. Two preparations of catalase were used. One was a commercial recrystallized ox-liver catalase (Worthington Biochemical Corp., St Louis, Mo., U.S.A.) with Kat.f. (Euler & Josephson, 1927) 26 000, and the other was a crystalline catalase (Kat.f. 58 000) prepared from human erythrocytes according to Herbert & Pinsent (1948). The concentration of the haemoprotein solutions was determined by the pyridine haemochromogen method of Keilin & Hartree (1951), and throughout this paper is expressed in terms of haemoprotein haematin.

Enzymic activities. Catalatic activity was estimated by the sodium perborate method of Feinstein (1949), at 37°, and the results were expressed in moles of perborate destroyed/μmole of catalase haematin under the conditions of the test. The concentration of catalase haematin required for this determination is of the order of 10^{-9}M. Kat.f. values were determined according to Euler & Josephson (1927). The rate of oxidation of ribose 5-phosphate (R 5-P) (Francoeur & Denstedt, 1954) was determined at pH 8·5, with 0·025M-2-amino-2-hydroxymethylpropane-1:3-diol–HCl (tris) buffer, with 0·02M-R 5-P as hydrogen donor and 0·002M-$K_3Fe(CN)_6$ as hydrogen acceptor in Thunberg tubes under N_2 at 37°. The reduction of the $K_3Fe(CN)_6$ was followed colorimetrically in a Klett–Summerson photoelectric colorimeter (filter 42) in which the Thunberg tubes could fit directly.

Spectrophotometry. Hilger Uvispek and Beckman DU spectrophotometers were used. Anaerobic spectra were measured in 1 cm. quartz cells fused to quartz Thunberg tubes.

Tissue preparations. Male albino rats (100 g.) of a local strain, bred randomly, were decapitated and the liver and kidneys were removed to cooled moist Petri dishes and weighed rapidly. Suspensions (1:5) were prepared in water with a Potter glass homogenizer (Umbreit, Burris & Stauffer, 1949). Erythrocyte haemolysates from rat blood obtained by heart puncture under ether anaesthesia, or from human blood, were prepared by defibrinating the blood with glass beads, washing the erythrocytes with 0·85% NaCl soln. five times on the centrifuge, haemolysing the cells with an equal volume of water and centrifuging

down the cell debris. All tissue preparations were made at 4° and used within 1 hr.

Materials. The yeast ribonucleic acid (RNA) and sperm deoxyribonucleic acid (DNA) were ordinary depolymerized commercial preparations. Similarly, the purines and pyrimidines, their derivatives and the R 5-P were commercial preparations not specially purified. R 5-P was obtained from the barium salt by passing it through a column of Dowex-50 (H^+ form).

Hydrogen peroxide was estimated by titration with 0·05 N-$KMnO_4$. Nitrogen was purified by passing the gas through a column of activated copper, deposited on kieselguhr, at 180° (Brauer, 1954).

RESULTS

Inhibition of the catalatic activity of tissue suspensions by 3-amino-1:2:4-triazole

Rat-liver and -kidney suspensions. Fig. 1 (*A*) and (*B*) show that the incubation at 37° of rat-liver and rat-kidney suspensions with 3-amino-1:2:4-triazole (AT), in O_2–N_2 (5:95), resulted in a rapid loss of catalatic activity. Within 2 hr. the residual catalatic activity was less than 5% of the original activity, whereas the tissue suspensions similarly incubated without AT showed practically no loss of activity. The addition of substances which, as will be shown below, could cause the inhibition of purified preparations of catalase by AT, such as R 5-P, RNA, ascorbic acid or cysteine, did not increase the rate of inhibition of catalase in these tissue suspensions.

Human- and rat-blood haemolysates. With blood haemolysates prepared from human and rat erythrocytes it could be shown that incubation with AT did not cause any diminution of the catalatic activity, in contradistinction to what happened with liver and kidney suspensions (Fig. 2). However, the addition of R 5-P or RNA, which could effectively diminish the catalatic activity of purified preparations of catalase in the presence of AT, resulted in the inhibition of the enzyme. Similar inhibitions could be obtained by dialysing the haemolysates against low concentrations of H_2O_2 in the presence of AT (see below).

Inhibition of the catalatic activity of purified preparations of catalase by 3-amino-1:2:4-triazole

Requirements for the inhibition of purified catalase by 3-amino-1:2:4-triazole. The incubation at 37° of a recrystallized preparation of ox-liver catalase with AT did not cause any significant inhibition of the catalatic activity of the preparation within 3–6 hr. At the concentration of AT used (0·02 M)

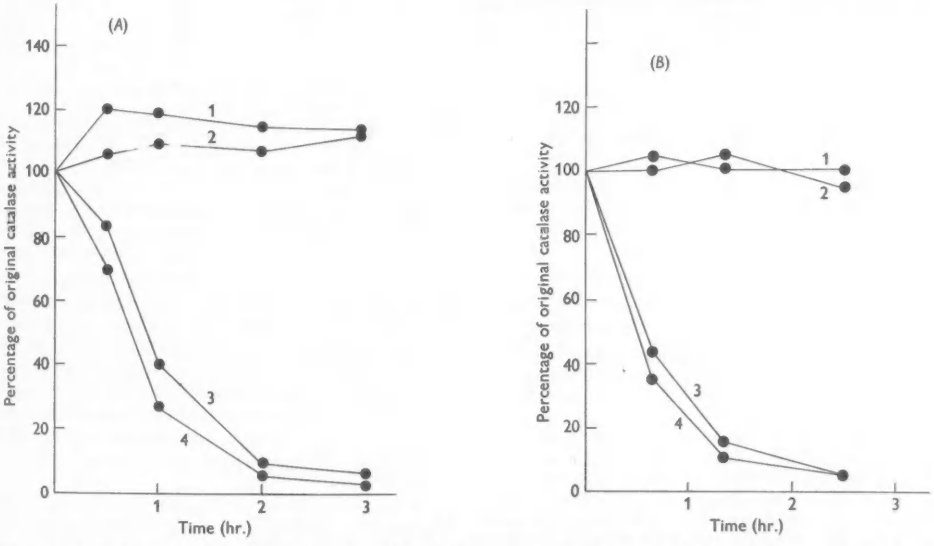

Fig. 1 (*A*). Effect of AT on the catalatic activity of rat-liver suspension. Final concentrations: 1:10 rat-liver suspension; 0·02 M-AT; 0·02 M-R 5-P; 0·02 M-$NaHCO_3$. Atmosphere O_2–CO_2–N_2 (5:5:90). pH 7·4. Total vol., 3 ml. Incubated at 37° in stoppered 25 ml. flasks. Liver suspension alone (1); liver suspension with R 5-P (2); liver suspension, R 5-P and AT (3); liver suspension with AT (4). (*B*). Effect of AT on the catalatic activity o rat-kidney suspension. Final concentrations and conditions were as for (*A*). Kidney suspension alone (1); kidney suspension with R 5-P (2); kidney suspension, R 5-P and AT (3); kidney suspension with AT (4).

Table 1. *Effect of various substances on the inhibition of purified ox-liver catalase by 3-amino-1:2:4-triazole*

Final concentrations in incubation mixtures were: $2{\cdot}3 \times 10^{-6}$ M-recrystallized ox-liver catalase haematin; 0·02 M-AT, 0·02 M added substances, unless otherwise stated. Total vol., 3 ml. Unless otherwise stated, the incubation was carried out at 37° under 5% of O_2 in N_2 with the addition of 5% of CO_2 for the bicarbonate buffer. Buffers: 0·02 M-$NaHCO_3$, pH 7·4; 0·025 M-tris buffer, pH 8·5; 0·05 M-phosphate buffer, pH 7. Concentrations of the RNA and DNA were calculated on the basis of the mol.wt. of the mononucleotides.

Added substance	pH	Percentage of original catalase activity after incubation for 2 hr.
R 5-P	7·4	5
R 5-P	8·5	5
R 5-P (under N_2)	8·5	99
Yeast RNA	7·4	17
Yeast RNA	8·5	5
Yeast RNA (incubation at 25°)	8·5	60
Yeast RNA (incubation at 0°)	8·5	95
Sperm DNA	7·4	9
Guanylic acid	7·4	29
Guanylic acid	8·5	25
Guanine	7·4	3
Ascorbic acid	8·5	8
Ascorbic acid (0·02 mM)	7·0	25
Ascorbic acid (0·02 mM) with EDTA (mM)	7·0	64
Cysteine	8·5	3
Cysteine (0·02 mM)	7·0	42
Cysteine (0·02 mM) with EDTA (mM)	7·0	101
Cytidylic acid	7·4	91
Adenosine 3-phosphate	7·4	91
Adenosine 5-phosphate	7·4	88
Adenosine 5-phosphate	8·5	102
Adenosine	7·4	89
Adenine	7·4	102
Ribose	7·4	83
Sodium perborate	8·5	86

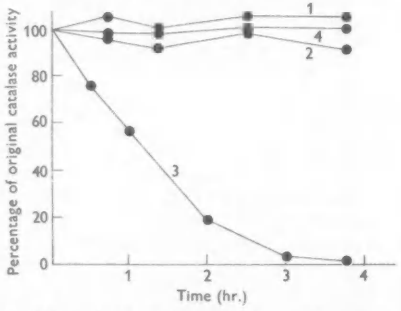

Fig. 2. Effect of AT on the catalatic activity of human-erythrocyte haemolysate. Final concentration of blood haemolysates: four times the volume of packed erythrocytes. Other concentrations and conditions were as in Fig. 1 (*A*). Blood haemolysate alone (1); blood haemolysate with R 5-P (2); blood haemolysate, R 5-P and AT (3); blood haemolysate with AT (4).

prolonged incubations (18–24 hr.) caused relatively small but consistent inhibitions, resulting in catalatic activities that were 65–80% of those of the control enzyme, incubated under the same conditions without AT. However, the addition of a number of substances to the incubation mixture led to a rapid progressive inhibition of activity, which was practically complete with 2 hr. (Table 1). Of the various substances tested the following were effective: R 5-P, ascorbic acid, cysteine, a commercial yeast RNA, a commercial sperm DNA, guanylic acid and guanine. With limiting concentrations of ascorbic acid and cysteine, the addition of ethylenediaminetetra-acetic acid (EDTA) to the incubation mixture resulted in smaller inhibitions than those obtained without the chelating agent (Table 1). The following substances were ineffective: adenosine 3-phosphate, adenosine 5-phosphate, adenosine, adenine, ribose, cytidylic acid, sodium perborate.

A second requirement for the rapid inhibition of catalase by AT was the presence of O_2 during the incubation. When the incubation was carried out under purified N_2 no inhibition was observed, and in a standard test the atmosphere was always O_2–N_2 (5:95) (Table 1).

Incubation at 0° prevented the inhibition of catalase by AT, whereas incubation at 37° was

effective and incubation at 25° gave intermediate values, with RNA as added substance (Table 1).

Similarly, Sugimura (1956) found that O_2 was required for the inhibition of catalatic activity in liver suspensions by AT, and that incubation at 0° was not effective.

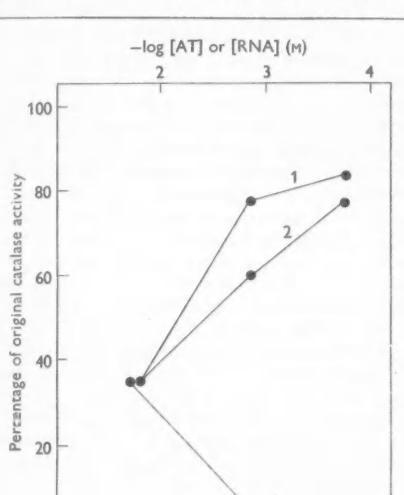

Fig. 3. Effect of the concentration of AT, yeast RNA and catalase on the inhibition of catalatic activity by AT. Curve 1: 2·3 μM-ox-liver catalase haematin, 20 mM-RNA and varying concentrations of AT. Curve 2: 2·3 μM-ox-liver catalase haematin, 20 mM-AT and varying concentrations of RNA. Curve 3: 20 mM-AT, 20 mM-RNA and varying concentrations of ox-liver catalase haematin. All flasks were incubated for 2 hr. at pH 8·5 (0·025 M-tris buffer) and 37° under O_2–N_2 (5:95). The concentration of RNA was calculated on the basis of the average mol.wt. of the mononucleotides.

Effect of the concentration of reactants on the inhibition of purified catalase by 3-amino-1:2:4-triazole. Fig. 3 shows the effect of the concentration of AT, RNA and catalase respectively on the rate of inhibition of the catalatic activity of recrystallized ox-liver catalase. It can be seen that the lower the concentration of AT and RNA and the higher the concentration of catalase, the smaller the inactivation rate.

Lack of effect of pre-incubation of catalase with ribose 5-phosphate, 3-amino-1:2:4-triazole or both on the inhibition of catalase by 3-amino-1:2:4-triazole. In order to test whether, during the incubation of catalase with R 5-P and AT, an excess of free inhibitor was produced or whether the incubation with R 5-P *per se* was necessary to enable the AT to combine with the enzyme, the following experiment was performed. Mixtures containing respectively catalase alone, AT alone, catalase and R 5-P, catalase and AT, and catalase, R 5-P and AT were incubated for 4 hr. under O_2–N_2 (5:95) at 37°. The catalatic activities of these incubation mixtures were determined. Equal quantities of them were then mixed together and the catalatic activity was determined at once. The results presented in Table 2 show that under these conditions there was no excess of free inhibitor produced during the incubation of catalase, R 5-P and AT that could inactivate either catalase or catalase pre-incubated with R 5-P. Moreover, catalase pre-incubated with R 5-P was not susceptible to an immediate action of AT. The inhibition of catalase by AT could be obtained only when all the required components were incubated together.

Sugimura (1956) was also unable to detect the presence of an inhibitory metabolite in incubation mixtures containing total liver suspensions and AT.

Table 2. *Lack of effect of pre-incubation of recrystallized ox-liver catalase with ribose 5-phosphate, 3-amino-1:2:4-triazole or both on the inhibition of catalase by 3-amino-1:2:4-triazole*

Final concentrations in mixtures pre-incubated were: $2·3 \times 10^{-6}$ M recrystallized ox-liver catalase haematin; 0·02 M-R 5-P; 0·02 M-AT; 0·025 M-tris buffer, pH 8·5. Total vol., 3 ml. Incubated for 4 hr. at 37°, under O_2–N_2 (5:95). At the end of the period of pre-incubation equal volumes of the original incubation mixtures were mixed, as indicated, and the catalatic activity was determined at once.

Mixtures pre-incubated	Catalatic activity (moles of perborate/μmole of catalase haematin) Before pre-incubation	After pre-incubation	Secondary mixtures	Catalatic activity (moles of perborate/μmole of catalase haematin) Expected, if no effect of pre-incubation	Observed
A. Catalase	37·2	35·5	*A* + *B*	35·5	32·5
B. AT	0	0	*A* + *E*	18·0	17·0
C. Catalase, R 5-P	34·2	30·5	*B* + *C*	30·5	30·0
D. Catalase, AT	34·7	31·0	*C* + *E*	15·5	15·5
			C + *D*	30·7	31·4
E. Catalase, R 5-P, AT	34·5	0·5	*D* + *E*	15·7	14·8

Inhibition in the presence of low and constant concentrations of hydrogen peroxide. The dialysis of H_2O_2 through a cellophan membrane has been used by Laser (1955) to obtain the gradual release of small amounts of H_2O_2 into a solution of catalase, thus obtaining high yields of the products of the peroxidatic activity of the enzyme. By the same principle, solutions containing either crystalline human-erythrocyte catalase (Fig. 4) or recrystallized ox-liver catalase, and AT at pH 6·8, were dialysed at 37° against small concentrations of H_2O_2 (0·2–2 mM). It was found that the inhibition of the catalatic activity proceeded as fast or faster than in total liver suspensions or with purified liver catalase together with substances mentioned above. Under the same conditions there was no significant inhibition in the absence of either AT or H_2O_2 within 3 hr. (Fig. 4). However, in the absence of H_2O_2, inhibitions of up to 50% could be obtained if the incubation was continued for 18 hr. When sodium perborate or H_2O_2 was added directly to solutions containing catalase and AT, the resulting inhibition was small and did not differ significantly from that obtained with AT alone (see Table 1).

Protective effect of ferricyanide on the inhibition of catalase by 3-amino-1:2:4-triazole. In the systems in which AT could cause the inhibition of catalase the addition of ferricyanide prevented this inhibition. Ferrocyanide had no effect. When the system RNA–AT–catalase in O_2–N_2 (5:95) was used, concentrations of ferricyanide of the order of mM were fully effective (Fig. 5). Experiments with the H_2O_2 dialysis technique, designed to show whether the protective effect of ferricyanide was accompanied by the formation of a ferricyanide–inhibitor complex leading to a change in the ferricyanide spectrum or whether ferricyanide was reduced to ferrocyanide, were consistently negative.

Spectral changes during the inhibition of purified ox-liver catalase by 3-amino-1:2:4-triazole. Concentrated solutions of recrystallized ox-liver catalase (final concentration in incubation mixture: 0·031 mM-haematin) were dialysed in the presence of AT (0·02M) against H_2O_2 (4 mM) at 37° and pH 6·8. Controls included catalase dialysed against H_2O_2 in the absence of AT, catalase dialysed against water in the presence of AT and catalase dialysed against water in the absence of AT. When after $2\frac{1}{2}$ hr. the catalatic activity of the solution con-

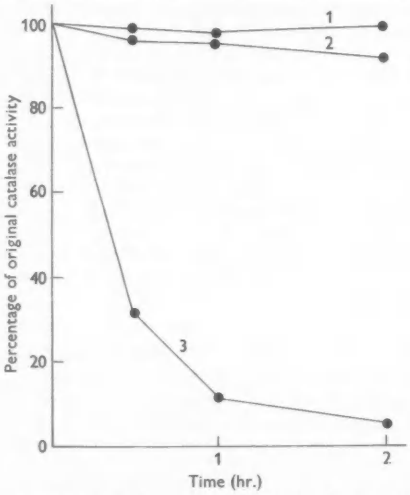

Fig. 4. Inhibition of crystalline human-erythrocyte catalase by dialysis of H_2O_2 into the catalase solution in the presence of AT. Final concentrations: 2·1 μM crystalline human-erythrocyte catalase haematin; 0·02M-AT; 0·033M-phosphate buffer, pH 6·8. Total volume of catalase-containing solution in dialysis bag: 6 ml. Outer solution (50 ml.) contained 2 mM-H_2O_2 with the same concentrations of AT and buffer as the solution in the dialysis bag. Temp., 37°. Catalase dialysed against H_2O_2 (1); catalase with AT dialysed against AT (2); catalase with AT dialysed against H_2O_2 and AT (3).

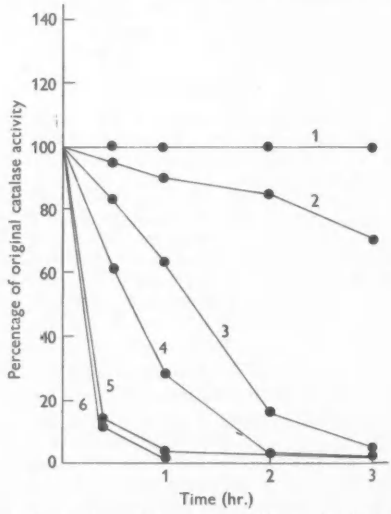

Fig. 5. Protective effect of ferricyanide on the AT inhibition of recrystallized ox-liver catalase in the presence of yeast RNA and O_2. Final concentrations: 2·3 μM-ox-liver catalase haematin; 0·02M-AT; 0·02M-RNA; 0·025M-tris buffer, pH 8·5; varying concentrations of $K_3Fe(CN)_6$ and $K_4Fe(CN)_6$. Total vol., 3 ml. Atmosphere O_2–N_2 (5:95). Temp., 37°. Ferricyanide concentrations: 1·28 mM (1); 0·385 mM (2); 0·128 mM (3); 0·0385 mM (4); 0·0128 mM (5). Ferrocyanide concentration: 5·2 mM (6). Concentration of the RNA was calculated on the basis of the average mol.wt. of the mononucleotides.

taining all three components had decreased to 3% of the original activity, the activity of the control solutions was unchanged. The spectrum of the contents of each dialysis bag was then recorded at once. The results are presented in Fig. 6 (*A*) and (*B*). There was no change in the spectrum of catalase incubated under the above conditions with either H_2O_2 or AT; catalase incubated with both H_2O_2 and AT showed a broadening of the α-band and a shift of its maximum towards the red end of the spectrum, as well as a slight decrease in the height of the Soret band. Under similar conditions, with appropriate concentrations of catalase and AT, it was not found possible to demonstrate any consistent changes in the concentration of AT, the spectrum of AT in the far ultraviolet region being used as a measure of its concentration.

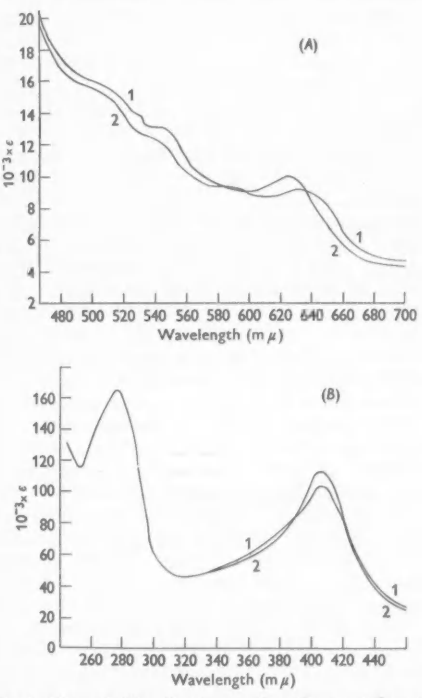

Fig. 6 (*A*) and (*B*). Spectrum of ox-liver catalase inhibited by AT. Recrystallized ox-liver catalase (final concn. 0·031 mM-catalase haematin) dialysed at 37° against H_2O_2 (2 mM) in the presence of AT (0·02 M) at pH 6·8 (0·033 M-phosphate buffer) for 2½ hr. (curve 1). At the end of the dialysis period the catalatic activity of the solution had decreased to 3% of its original value. Controls included catalase solutions dialysed under the same conditions as above, against water in the absence of AT, against water in the presence of AT and against H_2O_2 in the absence of AT (curve 2). At the end of the dialysis period the spectra of all three controls were identical and their catalatic activity had not varied significantly from the original value.

Properties of the catalase inhibited by 3-amino-1:2:4-triazole

Preparation of inhibited catalase. Catalase inhibited by AT was prepared by incubating at 37° recrystallized ox-liver catalase (0·031 mM) at pH 8·5 with R 5-P and AT (both 0·02 M) in O_2–N_2 (5:95), or by dialysing at 37° H_2O_2 (4 mM) into a solution of catalase and AT. When inhibition was complete (3–4 hr.) the preparations were dialysed against 0·85% NaCl soln. (changed twice daily) for 5 days to get rid of the excess of reagents. In order to crystallize the inhibited enzyme these solutions were then dialysed against glass-distilled water (changed daily). The crystals obtained, examined under the microscope with ordinary and polarized light, appeared to be identical with those of the original uninhibited ox-liver catalase.

Spectrum of inhibited catalase. The spectrum of the preparations of catalase inhibited by AT was identical with that given in Fig. 6 (*A*) and (*B*). Addition of H_2O_2 to these preparations under an atmosphere of pure N_2 did not cause any change in the spectrum.

Enzymic activity of inhibited catalase. The preparations of catalase inhibited by AT were devoid of any detectable catalatic activity in concentrations 100 times those used for testing the original preparation. However, when tested for the oxidation of R 5-P with $K_3Fe(CN)_6$ as hydrogen acceptor (Francoeur & Denstedt, 1954), as given under Methods, these preparations showed the full activity of the original uninhibited catalase. During the oxidation of R 5-P the inhibited catalase did not recover its catalatic activity, remaining fully inhibited, whereas the control uninhibited enzyme retained its full catalatic activity (Table 3).

Lack of reversal of inactivation of inhibited catalase by dialysis. Dialysis of preparations of ox-liver catalase inhibited by AT against 0·85% NaCl soln. (changed daily) for 15 days did not result in any increase of the catalatic activity of the preparation.

DISCUSSION

The experiments described above showed that the presence of hydrogen peroxide was required for the inhibition of catalase by 3-amino-1:2:4-triazole. In the absence of hydrogen peroxide only a relatively small degree of inhibition could be obtained after prolonged incubation. The inhibition observed was of the type described by Heim *et al.* (1956) after the injection of 3-amino-1:2:4-triazole into rats, and not the one obtained by these authors with high

Table 3. *Oxidation of ribose 5-phosphate by AT-inhibited catalase, with potassium ferricyanide as hydrogen acceptor*

Final concentrations in pre-incubation mixtures were: 0·46 μM recrystallized ox-liver catalase haematin; 0·02 M-R 5-P; 0·02 M-AT; 0·025 M-tris buffer, pH 8·5. Pre-incubation was for 2 hr. at 37° under O_2–N_2 (5:95). Total vol., 6 ml. Final concentrations in R 5-P oxidation test mixture: 0·384 μM-catalase haematin; 0·016 M-R 5-P; 0·002 M-$K_3Fe(CN)_6$; 0·025 M-tris buffer, pH 8·5. Oxidation of R 5-P carried out after pre-incubation in Thunberg tubes, under N_2, at 37°.

Pre-incubation mixture	Catalatic activity (moles of perborate/μmole of catalase haematin)		R 5-P oxidation rate after pre-incubation [m-moles of $K_3Fe(CN)_6$ reduced/hr./ μmole of catalase haematin]	Catalatic activity after R 5-P oxidation (moles of perborate/μmole of catalase haematin)
	Before incubation	After incubation		
A. Catalase, R 5-P	28·5	25·3	2·11	25·0
B. Catalase, R 5-P, AT	29·0	1·7	2·16	1·4

concentrations of 3-amino-1:2:4-triazole *in vitro*, since it could not be reversed by prolonged dialysis. The effect of substances other than hydrogen peroxide which were found to be effective in causing the inhibition of catalase by 3-amino-1:2:4-triazole, such as ribose 5-phosphate, nucleic acids, ascorbic acid and cysteine, required the presence of oxygen, and therefore probably acted by producing small amounts of hydrogen peroxide by auto-oxidation. This conclusion was further supported by the fact that with ascorbic acid and cysteine the addition of ethylenediaminetetra-acetic acid which, like other chelating agents, inhibits the auto-oxidation of these compounds, was found to decrease considerably their effectiveness in causing the inhibition of catalase by 3-amino-1:2:4-triazole.

The inhibition of catalase by 3-amino-1:2:4-triazole could thus be due either to the oxidation of 3-amino-1:2:4-triazole by catalase–hydrogen peroxide to an inhibitory metabolite under conditions favouring the peroxidatic activity of the enzyme (Laser, 1955), or to the reaction of unchanged 3-amino-1:2:4-triazole with the enzyme under these conditions. It has so far not been possible to eliminate either of these two possibilities. The absence of an excess of free inhibitor in incubation mixtures containing catalase, 3-amino-1:2:4-triazole and hydrogen peroxide, and the slow partial inhibition of catalase by 3-amino-1:2:4-triazole in the absence of hydrogen peroxide (see also Heim *et al.* 1956), seem to favour the second mechanism, but cannot be considered as conclusive evidence against the first. It should be noted that both possible mechanisms proposed for the rapid inhibition of catalase by 3-amino-1:2:4-triazole require hydrogen peroxide. In other cases in which hydrogen peroxide has been shown to play a role in the inhibition of the enzyme, such as with azide and hydroxylamine, an inhibitor (nitric oxide) is produced by the peroxidatic action of the enzyme, and the original substances are themselves strong immediate inhibitors (Keilin & Hartree, 1954). With 3-amino-1:2:4-triazole, even in the presence of hydrogen peroxide, the inhibition occurred much more slowly.

Catalase inhibited by 3-amino-1:2:4-triazole showed a spectrum somewhat different from that of catalase, with a shift of the visible part of the spectrum towards the longer wavelengths and a slight decrease of the height of the Soret band. Similarly, the spectrum of azide–catalase is not very different from that of the original catalase, but when hydrogen peroxide is added and nitric oxide–catalase is formed there is a gross change in the visible spectrum (Keilin & Hartree, 1945). No such effect could be observed on the addition of hydrogen peroxide under anaerobic conditions to catalase inhibited by 3-amino-1:2:4-triazole.

In the presence of oxygen, but in the absence of added hydrogen peroxide, the effect of 3-amino-1:2:4-triazole on the catalatic activity of tissue suspensions exactly paralleled its effect *in vivo*, described by Heim *et al.* (1956). The catalatic activity of liver and kidney suspensions was rapidly reduced to low levels, whereas that of blood haemolysates was unaffected. The catalatic activity of blood haemolysates could, however, be inhibited if hydrogen peroxide was added under suitable conditions. Purified erythrocyte catalase itself did not differ from purified liver catalase in its behaviour towards 3-amino-1:2:4-triazole. Thus the inhibition of liver and kidney catalase by 3-amino-1:2:4-triazole administered to rats, and its lack of effect on blood catalase (Heim *et al.* 1956), as well as the corresponding effects in tissue suspensions, may possibly be ascribed to the availability of sufficient hydrogen peroxide in liver and kidney and its lack in blood. This does not, however, preclude the possibility that other factors in blood

interfere with the reaction of catalase, hydrogen peroxide and 3-amino-1:2:4-triazole just as ferricyanide was shown to do.

SUMMARY

1. 3-Amino-1:2:4-triazole caused a complete, irreversible and relatively rapid inhibition of crystalline purified preparations of liver and erythrocyte catalase in the presence of low and constant concentrations of hydrogen peroxide. Without hydrogen peroxide the inhibition of catalase by 3-amino-1:2:4-triazole was slight and proceeded much more slowly. Other substances that could replace hydrogen peroxide required the presence of oxygen, and it is considered that they acted by producing peroxide by auto-oxidation.

2. Ferricyanide, but not ferrocyanide, prevented this inhibition.

3. The spectrum of inhibited catalase differed from that of catalase. The visible part of the spectrum was shifted towards the longer wavelengths, and the Soret band was slightly lowered.

4. Inhibited catalase was obtained in a crystalline form that did not differ from that of uninhibited catalase.

5. The ability of catalase inhibited by 3-amino-1:2:4-triazole to oxidize ribose 5-phosphate with potassium ferricyanide as hydrogen acceptor was unchanged.

6. In the presence of oxygen, but in the absence of added hydrogen peroxide, 3-amino-1:2:4-triazole caused the inhibition of catalase in liver and kidney suspensions, but had no effect in blood haemolysates. The catalatic activity of blood haemolysates could be inhibited by 3-amino-1:2:4-triazole only in the presence of added hydrogen peroxide.

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The Kinetics of Hydrazinolysis of Simple Peptides in Anhydrous Hydrazine

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The method of Akabori, Ohno & Narita (1952) for the determination of the *C*-terminal amino acids of peptides and proteins consists in treating with anhydrous hydrazine at 100–120° for about 8 hr. to split the peptide bonds and produce amino acid hydrazides and free amino acids from the *C*-terminal positions. The latter can be separated from the former by treatment with benzaldehyde, by dinitrophenylation and subsequent extraction with sodium bicarbonate of the dinitrophenyl-amino acid from the dinitrophenyl-amino acid hydrazide (Ohno, 1953). The main disadvantage of this method is that the severe treatment required for complete hydrazinolysis causes decomposition of cysteine and cystine and poor yields of arginine, aspartic acid and glutamic acid (Locker, 1954; Niu & Fraenkel-Conrat, 1955). The problem of severity of treatment has been largely overcome by the addition of an acid catalyst, hydrazine sulphate, to the reaction mixture, when heating at 60° for 16 hr. brings about complete fission of the peptide bond (in the seven peptides studied) and allows all the common amino acids to be recovered in good yields (Bradbury, 1956).

In order to apply the hydrazine method with confidence it is important to know more about the

hydrazinolysis reaction. One requires to know the rates of the acid-catalysed and uncatalysed reaction for various types of peptide bonds at different temperatures and the influence of water on the system. Such information would be useful in work on the identification of small peptides from partial hydrolysates (Kay, Schroeder, Munger & Burt, 1956; Kroner, Tabroff & McGarr, 1955). It would also be of importance in deciding the appropriate conditions of hydrazinolysis of proteins in order to obtain on the one hand the maximum yield of *C*-terminal amino acid and no appreciable amount of *C*-terminal peptide (Bradbury, 1958), or, on the other, the maximum yield of *C*-terminal peptides [if one is interested in the *C*-terminal sequence of amino acids (Niu & Fraenkel-Conrat, 1955; Ohno, 1955)].

Information of this type was obtained by a study of the kinetics of the acid-catalysed and uncatalysed hydrazinolysis of the peptides glycylglycine, DL-leucylglycine, L-lysylglycine dihydrobromide, L-leucyl-DL-valine and diglycylglycine in anhydrous hydrazine. First-order rate constants were obtained in all cases by use of large excesses of hydrazine and hydrazine sulphate over the amount of peptide present. Energies and entropies of activation were calculated in two cases. As this is probably the first example of kinetic measurements in anhydrous hydrazine as a solvent, some comparison is made between these results and those obtained for the similar case of acid hydrolysis in water. A subsequent paper deals with the application of the hydrazine method to the determination of the *C*-terminal amino acids of proteins.

EXPERIMENTAL

Materials

Peptides. Glycylglycine (British Drug Houses Ltd., Poole, Dorest), DL-leucylglycine and diglycylglycine (L. Light and Co. Ltd., Colnbrook, Bucks) were used without further purification. A sample of ϵ-*N*-benzyloxycarbonyllysylglycine ethyl ester (supplied by Dr J. M. Swan, Wool Textile Research Laboratory, Melbourne) was hydrolysed by treatment with N-NaOH in acetone at room temperature for 1 hr., acidified and the free acid filtered off and dried (m.p. 204°, uncorr.). This was allowed to react with a small volume of 20% (w/w) HBr in acetic acid at room temperature for 30 min.; the concentration of HBr is not critical. Lysylglycine dihydrobromide was precipitated by the addition of dry ether, filtered off, washed with ether and dried *in vacuo* at 60°. Equivalent weight: theory, 182·4; found, 193. L-Leucyl-DL-valine was kindly prepared by Dr W. E. Savige, Wool Textile Research Laboratory, Melbourne. The purity of these peptides was checked by dinitrophenylation followed by one-dimensional paper chromatography; no spots due to impurities were observed.

Anhydrous hydrazine. Hydrazine hydrate (100%), supplied by Gollin and Co., Melbourne, was dried over CaO and distilled at room temperature and 10^{-4} mm. Hg pressure to give a product containing 95% of N_2H_4 (Penneman & Audrieth, 1948). After a second drying and distillation the analysis was $100 \pm 0{\cdot}3$% of N_2H_4.

Preparation, separation and estimation of dinitrophenyl-peptides and dinitrophenyl-amino acids

Amino acids and peptides were dinitrophenylated by at least one of the methods due to Sanger (1945) and Levy (1954) with some slight modifications. In the first method 5 mg. of the amino acid or peptide was dissolved in about 5 ml. of 0·5M-$NaHCO_3$ soln., 5 ml. of a solution of 1-fluoro-2:4-dinitrobenzene (FDNB) in absolute ethanol (27 mg./ml.) was added and sufficient ethanol to make the ratio of ethanol to water 2:1. The solution was shaken for 3 hr. at room temperature, the alcohol evaporated and 5 ml. of 0·5M-carbonate buffer, pH 9, added. The aqueous solution was extracted with ether (4 × 5 ml.) and the combined ether layers were washed once with carbonate buffer, which was added to the aqueous solution. The latter was acidified and extracted with 5 ml. portions of ethyl acetate until these washings were colourless. In general five washings were sufficient, but in some cases, e.g. dinitrophenyl (DNP)-diglycylglycine, 10–15 washings were necessary. In the second method 5 mg. of the amino acid or peptide was dissolved in about 6 ml. of 0·5M-carbonate buffer, pH 9, and about 50 mg. of FDNB added. The mixture was stirred at 40° for 3 hr., after which it was extracted by the method already described.

The ethyl acetate solution of the DNP-amino acid or DNP-peptide obtained by either method was made up to volume in a standard flask and a portion applied to the paper with a micrometer syringe. The spots were run in one dimension with the toluene, phosphate or 2-methylbutan-2-ol solvent (Fraenkel-Conrat, Harris & Levy, 1955). The paper was dried, the spots together with three blanks were cut out, weighed approximately, eluted with 5 ml. of 2% $NaHCO_3$ soln. at 50° for 15 min. and their optical density was measured at 360 mμ (385 mμ for DNP-proline) in a 1 cm. cell of the Unicam spectrophotometer. The small blank correction (usually about 0·01/100 mg. of paper) was subtracted from the observed optical density for calculation of the molar-extinction coefficient. Solutions of these materials in ether, ethyl acetate or acetone have been stored in the refrigerator, without noticeable decomposition, for more than a year.

The extinction coefficients obtained by the above methods with one-dimensional chromatography are summarized in Table 1. They have been used in previous work (Bradbury, 1956) and in the present investigation. The average value of $1{\cdot}47 \times 10^4$ for the 17 DNP-amino acids listed is about 20% lower than that obtained for the pure DNP-amino acids in $NaHCO_3$ soln. (Fraenkel-Conrat *et al.* 1955). The difference is attributed to incomplete dinitrophenylation, losses during extraction and losses on the paper during chromatography. However, as these sources of error are reproduced in later hydrazinolysis experiments it is reasonable to assume that they are cancelled out by the use of the above values of ϵ.

It was not possible to dinitrophenylate glycylglycine or diglycylglycine quantitatively by Levy's (1954) method and in this respect these two peptides seem to be anomalous, since a number of other dipeptides (some not included in Table 1) reacted quite satisfactorily under these conditions. However, on prolonged treatment at pH 9 at

Table 1. *Molar-extinction coefficients of dinitrophenyl-amino acids and dinitrophenyl-peptides*

For explanation of the abbreviations used for amino acids in this Table see *Biochem. J.* (1953), **55**, 5.

Method of dinitrophenylation	Extinction coefficient × 10^{-4}		
	Levy (1954) (pH 9 40°)	Sanger (1945) (pH about 8)	Sanger (1945) ($NaHCO_3$ replaced by 0·4 M-phosphate buffer, pH 6·4)
DNP-amino acid or DNP-peptide Ala; Arg; Asp; $CySO_3H$; Glu; Gly; His (bis); His (α-mono-); Leu; Met; Pro; Phe; Ser; Thr; Try; Tyr (bis); Val	1·47 ± 0·03	—	—
L-Cystine (bis)	2·28	—	—
L-Lysine (bis)	2·20	—	—
Glycine	1·49	1·40	0·52 (variable)
Glycylglycine	0·45 (variable)	1·14	1·07
Diglycylglycine	0·48* (variable)	1·08	1·08
DL-Leucylglycine	1·45	1·51	—
L-Leucyl-DL-valine	—	1·30	—
Lysylglycine (bis)	—	2·0	—

* Obtained $\epsilon = 1{\cdot}07$ on shaking at room temperature in ethanol–water (2:1) for 66 hr. with buffer, pH 9, and FDNB.

room temperature in the mixed solvent, a quantitative yield of DNP-diglycylglycine was obtained. It is interesting to note that glycylglycine and diglycylglycine with pK_2 8·0 can be dinitrophenylated quantitatively at pH 6·4, whereas glycine (pK_2 9·6) reacted only partially. This behaviour suggests that FDNB reacts only with the uncharged form of the amino group.

Procedure for rate measurements

To a small amount of peptide (usually 2 mg.) was added 30 times the weight of hydrazine sulphate (AnalaR) and the materials were dried for about 3 hr. at 10^{-4} mm. Hg pressure. An accurately known volume of anhydrous hydrazine was distilled in from a calibrated reservoir at room temperature, and the tube sealed and heated under vacuum for the appropriate time and temperature (±0·2°). It was then opened, excess of hydrazine removed in a vacuum desiccator over conc. H_2SO_4 and the residue dissolved in hot water and shaken with about 0·2 ml. of benzaldehyde (AnalaR) for 2 hr. to remove hydrazine sulphate and amino acid hydrazides as the benzal compounds. The mixture was centrifuged, the benzaldehyde layer washed twice with water, the aqueous layers were dinitrophenylated by Sanger's (1945) method and the alcohol was removed at the pump and 5 ml. of carbonate buffer, pH 9, added. This solution was extracted with 4 × 5 ml. portions of ether–ethyl acetate (1:1, v/v); the organic layers were diluted with 20 ml. of ether and extracted with 3 × 10 ml. of buffer, pH 9. The combined aqueous layers were acidified with HCl and extracted with 5 ml. portions of ethyl acetate until the washings (usually five) were colourless. The solution was made up to volume in a standard flask and at least two portions were run on a paper chromatogram with a suitable solvent to separate the unchanged peptide from the *C*-terminal amino acid and the other products of reaction. The spots were cut out and measured as previously described and the percentage yield was calculated with the extinction coefficients of Table 1.

The extraction procedure described above is simpler than the three-stage process used by Ohno (1953) and Niu & Fraenkel-Conrat (1955) or the rather involved procedures recently described by Akabori *et al.* (1956). It has the additional advantage over the former that the bis-DNP-amino acids of tyrosine and cysteine, which are not completely recovered by their method [it involves extraction from ether–ethyl acetate (1:1, v/v) by $NaHCO_3$ soln.], are extracted quantitatively from ether–ethyl acetate (3:1, v/v) by buffer solution, pH 9.

RESULTS

Kinetic analysis

The equation for the reaction between a dipeptide and anhydrous hydrazine is given by reaction 1, Table 2. Since the concentration of the hydrazine does not alter during the reaction the rate of reaction is proportional to the concentration of dipeptide present. Integration of the simple first-order rate equation gives

$$k_u t = 2{\cdot}303 \log c_0/c, \qquad (1)$$

where c_0 is initial concentration of dipeptide and c is concentration of dipeptide at time t. k_u is calculated from the slope of the straight line obtained by plotting $\log c$ against time and the second-order rate constant k''_u by the equation $k''_u = k_u/[N_2H_4]$.

In the presence of hydrazine sulphate, which reacts with hydrazine according to the equation $N_2H_4,H_2SO_4 + N_2H_4 = 2N_2H_5^+ + SO_4^{2-}$ (Audrieth & Ogg, 1951), reactions 2 and 3 (Table 2) occur simultaneously. By using a large excess of acid catalyst over the amount of peptide present reaction 3 becomes first-order.

Table 2. *Summary of hydrazinolysis reactions and their rate constants*

Reaction no.	Equation	Rate constants: Pseudo-first-order	Rate constants: Second-order
	Dipeptide		
1	$H_2N{\cdot}CHR_1{\cdot}CO{\cdot}NH{\cdot}CHR_2{\cdot}CO_2H^* + N_2H_4 = H_2N{\cdot}CHR_1{\cdot}CO{\cdot}NH{\cdot}NH_2 + H_2N{\cdot}CHR_2{\cdot}CO_2H$	k_u	k_u
2	$H_2N^+{\cdot}CHR_1{\cdot}CO{\cdot}NH{\cdot}CHR_2{\cdot}CO_2H + N_2H_4 = H_2N^+{\cdot}CHR_1{\cdot}CO{\cdot}NH{\cdot}NH_2 + H_2N{\cdot}CHR_2{\cdot}CO_2H$	k'_u	—
3	$H_3N^+{\cdot}CHR_1{\cdot}CO{\cdot}NH{\cdot}CHR_2{\cdot}CO_2H + N_2H_5^+ = H_3N^+{\cdot}CHR_1{\cdot}CO{\cdot}NH{\cdot}NH_2 + H_3N^+{\cdot}CHR_2{\cdot}CO_2H$	k_c	k''_c
	Diglycylglycine (g_3)		
4	$g_3 + N_2H_4 = H_2N{\cdot}CH_2{\cdot}CO{\cdot}NH{\cdot}NH_2 + g_2$	k_{1u}	—
5	$g_2 + N_2H_4 = H_2N{\cdot}CH_2{\cdot}CO{\cdot}NH{\cdot}NH_2 + g$	k_{2u}	—
6	$g_3 + N_2H_4 = H_2N{\cdot}CH_2{\cdot}CO{\cdot}NH{\cdot}CH_2{\cdot}CO{\cdot}NH{\cdot}NH_2 + g$†	k_{3u}	—

* This may exist as $H_3N^+{\cdot}CHR_1{\cdot}CO{\cdot}NH{\cdot}CHR_2{\cdot}CO_2^-$ in anhydrous hydrazine.

† The further reaction $H_2N{\cdot}CH_2{\cdot}CO{\cdot}NH{\cdot}CH_2{\cdot}CO{\cdot}NH{\cdot}NH_2 + N_2H_4 = 2\,gNH{\cdot}NH_2$ is of no interest here since it cannot be measured.

The subscripts u and c denote the uncatalysed and catalysed reactions respectively; g, glycine; g_2, glycylglycine.

Table 3. *Uncatalysed hydrazinolysis of glycylglycine and* DL-*leucylglycine at 60°*

Time (hr.)	Glycine (%)	Dipeptide remaining (%)	Total	(Dipeptide %) corr., assuming total = 100 %	log (Dipeptide %) corr.
		Glycylglycine			
0	0	100	100	100	2·000
0·167	22·9	91·0	113·9	$\frac{91{\cdot}0 \times 100}{113{\cdot}9} = 80{\cdot}0$	1·903
0·50	40·2	67·0	107·2	62·7	1·797
1·50	56·0	39·7	95·7	41·5	1·618
4·00	99·0	4·8	103·8	4·6	0·670
		DL-Leucylglycine			
0	0	100	100	100	2·000
16·25	33·3	60·4	93·7	64·5	1·810
24	33·6	39·3	72·9	54·0	1·732
30	48·1	22·2	70·3	31·6	1·501

These two reactions then satisfy the equation

$$-dc/dt = (k'_u + k_c)\,c, \qquad (2)$$

where c is concentration of dipeptide at any time t. Integration of this first-order rate equation gives an equation similar to equation (1) in which $(k'_u + k_c)$ is calculated from the slope of a graph of log (% dipeptide) against time. Since $k'_u \simeq k_u$ (see below), k_c is calculated by subtraction and k''_c by the equation $k''_c = k_c/[N_2H_5^+]$.

The kinetics of the uncatalysed hydrazinolysis of diglycylglycine are complicated by the fact that there are two peptide bonds and hence three possible reactions (reactions 4–6, Table 2) as compared with only one (reaction 1) for the dipeptide. The rate of removal of diglycylglycine ($-dg_3/dt$) is given by the equation

$$-dg_3/dt = (k_{1u} + k_{3u})\,g_3,$$

where g_3 is concentration of diglycylglycine at any time t. The sum of the uncatalysed rate constants $(k_{1u} + k_{3u})$ can be obtained as described above. Similarly, one can write differential equations describing the rate of formation of glycylglycine (g_2) and glycine (g) and these can be integrated to give more complicated equations relating k_{1u}, k_{2u} and k_{3u} with measurable quantities. However, it has not been possible to refine the experimental technique sufficiently to obtain reasonable values for the separate rate constants k_{1u} or k_{3u}. The rate constant k_{2u} is obtained from the uncatalysed hydrazinolysis of glycylglycine. The hydrazinolysis of diglycylglycine in the presence of hydrazine sulphate involves the three rate constants for the uncatalysed reactions k_{1u}, k_{2u}, k_{3u} and three more for the analogous catalysed reactions k_{1c}, k_{2c}, k_{3c}. By use of the same treatment as above one obtains the sum $(k_{1u} + k_{3u} + k_{1c} + k_{3c})$ from the rate of disappearance of diglycylglycine and, since $(k_{1u} + k_{3u})$ is known, $(k_{1c} + k_{3c})$ is obtained by subtraction.

Uncatalysed reaction

The detailed results for the hydrazinolysis of glycylglycine and DL-leucylglycine at 60° are given in Table 3. As the experimental error involved in

Table 4. *Rate data for the uncatalysed hydrazinolysis of peptides*

Peptide	% water in N_2H_4 (w/w)	$10^3\ k''_u$ l.mole^{-1} min.$^{-1}$ at the temperatures stated 40°	60°	80°	100°	E (kcal. mole^{-1})	$-\Delta S^*$ cal. deg.$^{-1}$ mole^{-1}
Diglycylglycine	0·0	—	0·58[a]	—	—	—	—
Glycylglycine	0·0	—	0·41	—	—	—	—
L-Lysylglycine dihydrobromide	0·0	—	0·032	—	—	—	—
DL-Leucylglycine	0·0	0·0025	0·018	0·064	—	17·8	36
DL-Leucylglycine	5·0	—	0·018[b]	—	—	—	—
DL-Leucylglycine	35·0	—	Approx. 0·0033[c]	—	—	—	—
L-Leucyl-DL-valine	0·0	—	<0·0003	0·0014	0·0057	17·8	44

[a] Value of $(k''_{1t} + k''_{2u})$.
[b] The amount of leucine was found to be 0·6% of the total leucylglycine after separation on a buffered Celite column at pH 6·5 with ether as the mobile phase (Perrone, 1951).
[c] 1·4% of leucine present.

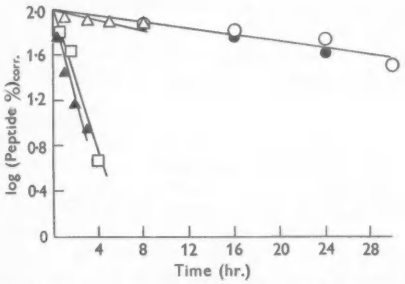

Fig. 1. Kinetics of uncatalysed hydrazinolysis at 60°. ○, Leu.Gly; △, Lys.Gly; □, Gly.Gly; ▲, Gly.Gly.Gly. All were in 100% N_2H_4. ●, Leu.Gly in 95% N_2H_4. For explanation of the abbreviations used for amino acids in this and subsequent figure legends see *Biochem. J.* (1953), **55**, 5.

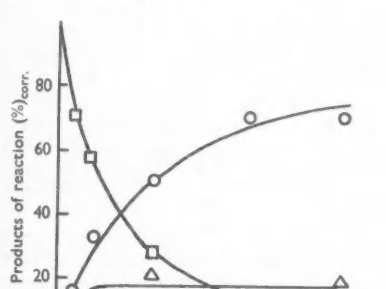

Fig. 2. Hydrazinolysis of diglycylglycine in anhydrous hydrazine at 60°. ○, Gly; △, Gly.Gly; □, Gly.Gly.Gly.

the determination of the percentage of glycine or of unchanged dipeptide is about ±10% (Bradbury, 1956), it is clear that variations of about ±20% in the total are to be expected. In some cases the total yield falls below 80%, owing to experimental losses which occur to some extent in the benzaldehyde treatment. These relatively large experimental errors make for inaccurate rate constants but the error can be reduced by the determination, in all cases, of the unchanged peptide and the amino acid produced by the reaction. In view of the known stability of these amino acids and peptides to the hydrazinolysis conditions it is reasonable to assume a 100% total recovery and to correct the figure for the percentage peptide on this basis (see Table 3). The first-order rate constant is then determined from the gradient of a graph of log (dipeptide %)$_{corr.}$ against time (see Fig. 1). In Table 4 are recorded the second-order rate constants k''_u, calculated by the equation $k''_u = k_u/[N_2H_4]$, together with the energies (E) and entropies (S) of activation calculated from the values of k''_u at different temperatures (Laidler, 1950). The behaviour of diglycylglycine during hydrazinolysis is shown in Fig. 2.

Catalysed reaction

All experiments were made in the presence of a large excess of hydrazine sulphate, hence first-order rate constants $(k'_u + k_c)$ were obtained in all cases. These are recorded in Table 5 for the hydrazinolysis of glycylglycine at three different concentrations of hydrazine sulphate. Assuming that $k'_u = k_u$, the value of k_c is calculated and also the second-order rate constant k''_c. The constancy of k''_c, within experimental error, confirms the validity of the assumption which is now used in the calculation of k''_c for the other peptides. These are summarized in Table 6, together with values of E and ΔS^* calculated from experiments at three

Table 5. *Effect of concentration of hydrazine sulphate on rate constants for glycylglycine at 60°*

$[N_2H_5^+]$	$10^3\ k_u$ (min.$^{-1}$)	$10^3\ (k'_u + k_c)$ (min.$^{-1}$)	$10^3\ k_c$ (min.$^{-1}$) assuming $k_u = k'_u$	$10^3\ k''_c = 10^3\ k_c/[N_2H_5^+]$ (l.mole^{-1} min.$^{-1}$)
0	12·4	—	—	—
0·50	—	25·4	13·0	26·0
1·00	—	47·5	35·1	35·1
2·00	—	72·4	60·0	30·0
				Mean 30·4±2·6

Table 6. *Rate data for the catalysed hydrazinolysis of peptides*

Peptide	Water in N_2H_4 (%, w/w)	$10^3\ k''_c$ (l.mole^{-1} min.$^{-1}$) 40°	60°	80°	100°	E (kcal. mole^{-1})	$-\Delta S^*$ cal. deg.$^{-1}$ mole^{-1}
Diglycylglycine	0·0	—	37·3[a]	—	—	—	—
Glycylglycine	0·0	—	30·4	—	—	—	—
L-Lysylglycine dihydrobromide	0·0	—	1·87	—	—	—	—
DL-Leucylglycine	0·0	0·38	1·45	4·9	—	13·5	40
DL-Leucylglycine	5·0	—	1·45[b]	—	—	—	—
DL-Leucylglycine	35·0	—	Approx. 0·9[c]	—	—	—	—
L-Leucyl-DL-valine	0·0	—	0·045	0·085	0·200	9·2	60

[a] Value of $k''_{1c} + k''_{3c}$. [b] Leucine content 0·8% of total leucylglycine. [c] Leucine content 1·3%.

Table 7. *Comparison of rate data for hydrazinolysis and hydrolysis*

Peptide	Hydrazinolysis at 60°: $10^3\ k''_u$ (l.mole^{-1} min.$^{-1}$)	$10^3\ k''_c$ (l.mole^{-1} min.$^{-1}$)	k''_c/k''_u	Hydrolysis in HCl at 99°*: $10^3\ k_2$ l.mole^{-1} min.$^{-1}$)
Diglycylglycine	0·58†	37·3‡	64	—
Glycylglycine	0·41	30·4	74	3·5
L-Lysylglycine dihydrobromide	0·0316	1·87	59	—
DL-Leucylglycine	0·0181	1·45	80	0·7
L-Leucyl-DL-valine	<0·0003	0·045	>150	—
L-Leucyl-D-leucine	—	—	—	0·2

* Harris, Cole & Pon (1956). † $(k''_{1u} + k''_{2u})$. ‡ $(k''_{1c} + k''_{3c})$.

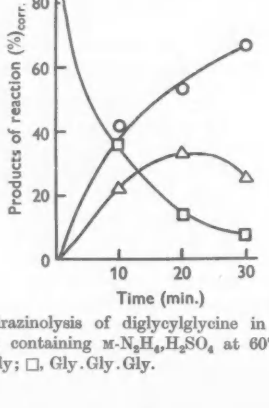

Fig. 3. Hydrazinolysis of diglycylglycine in anhydrous hydrazine containing M-N_2H_4,H_2SO_4 at 60°. ○, Gly; △, Gly.Gly; □, Gly.Gly.Gly.

different temperatures. Fig. 3 shows the course of the catalysed hydrazinolysis of diglycylglycine at 60° in M-hydrazine sulphate.

DISCUSSION

From Table 7 it is seen that, for the peptides studied, $k''_c/k''_u < 59$, hence the overall rate of reaction at 60° in M-hydrazine sulphate ($[N_2H_5^+]=2$) is up to five times as fast:

$$1 + (k''_c.[N_2H_5^+]/k''_u.[N_2H_4]) = 1 + (59 \times 2/30{\cdot}4) \simeq 5$$

as in the absence of catalyst. It is therefore advantageous to use an acid catalyst in determinations of the *C*-terminal amino acids of a peptide or protein by the hydrazine method in order to obtain the maximum amount of peptide bond breakage with the minimum decomposition of the *C*-terminal amino acid (Bradbury, 1956). Thus the treatment in anhydrous hydrazine containing

Table 8. *Summary of energy and entropy of activation data*

Peptide	E (kcal. mole^{-1})		$-\Delta S^*$ (cal. deg.$^{-1}$ mole^{-1})	
	Catalysed	Uncatalysed	Catalysed	Uncatalysed
DL-Leucylglycine	13·5	17·8	40	36
L-Leucyl-DL-valine	9·2	17·8	60	44

M-hydrazine sulphate at 60° for 16 hr. leaves only 4% of DL-leucylglycine but over 90% of L-leucyl-DL-valine unreacted. Since DL-leucylglycine is a fairly stable peptide this treatment should be suitable for most proteins and is adequate for insulin and lysozyme (Bradbury, 1958). For a protein with a *C*-terminal sequence such as valylleucine mild treatment at 60° would liberate a large amount of a *C*-terminal peptide which could be identified. This would be preferable to treatment for 100 hr. at 100°, which would be necessary in order to liberate valine completely but would cause some decomposition of the *C*-terminal amino acid and also increase the amount of non-*C*-terminal amino acids produced by side reactions (Bradbury, 1958).

It is found that there is no increase of the amount of the non-*C*-terminal amino acid leucine from DL-leucylglycine when hydrazinolysis is carried out in the presence of 5% of water. Even when 35% of water is present the leucine content is only doubled, although the rates of both the catalysed and uncatalysed reactions are markedly decreased. These results are in good agreement with those obtained with insulin (Bradbury, 1958), and show that there is no need to take elaborate precautions to exclude water during hydrazinolysis. At least 5% of water can be tolerated [Niu & Fraenkel-Conrat (1955) state that up to 10% does not cause appearance of non-*C*-terminal amino acids] but hydrazine hydrate as used by Schlogl, Wessely & Wawersich (1954) should be avoided.

Lawrence & Moore (1951) found that in the acid hydrolysis of diglycylglycine the first peptide bond reacted about eight times as fast as in the corresponding hydrolysis of glycylglycine. This has been interpreted as due to stabilization of the peptide bond of glycylglycine owing to repulsion between the adjacent NH_3^+ group and the attacking H_3O^+ ion (Harris, Cole & Pon, 1956). Such an electrostatic effect cannot be operative in the acid-catalysed hydrazinolysis of glycylglycine since the sum of the rate constant ($k''_{1c}+k''_{3c}$) for diglycylglycine is less than twice k''_c for glycylglycine (the value expected if both peptide bonds in diglycylglycine had the same reactivity as the one in glycylglycine).

The prime importance of the steric effect in the hydrazinolysis reaction is shown by consideration of the results for energy and entropy of activation summarized in Table 8. The approximately 40-fold decrease of the rate constant in going from DL-leucylglycine to L-leucyl-DL-valine (for either the catalysed or uncatalysed reaction) is reflected mainly in the decrease of the entropy of activation. This means that there is increased steric hindrance of reaction due to the introduction of the bulky *iso*propyl side chain of valine in place of the hydrogen atom of glycine. The same effect, on a greatly reduced scale, is noted in the hydrolysis of glycylglycine and glycyl-L-leucine, where the introduction of the bulky *iso*butyl side chain of leucine sterically hinders the reaction (Lawrence & Moore, 1951).

It is interesting to compare the relative rates of the acid-catalysed hydrazinolysis and hydrolysis reactions for various peptides. From Table 7 it is seen that in going from glycylglycine to DL-leucylglycine there is a 21-fold decrease of the rate constant of hydrazinolysis but only a fivefold decrease for hydrolysis. An even greater contrast is obtained between DL-leucylglycine and L-leucyl-DL-valine (L-leucyl-D-leucine in hydrolysis can be taken as similar to the latter) where there is a 32-fold decrease in rate constant of hydrazinolysis and only 3·5 for hydrolysis. This difference of behaviour is mainly due to steric factors, which are much more important in hydrazinolysis than in hydrolysis. This is only to be expected since a simple calculation shows that at 50° a water molecule occupies 30·3 Å^3 compared with 54·2 Å^3 for a molecule of hydrazine. The ionic volumes will be somewhat larger but it is clear that it is more difficult for the bulkier $N_2H_5^+$ group to approach the reactive centre than for the much smaller H_3O^+ ion.

The wide range of reactivity of various peptide bonds towards hydrazine, if reproduced in the protein molecule, may allow the selective fission of peptide bonds by careful control of the reaction conditions. A possible application would be an attempt to elucidate amino acid sequences of proteins by partial hydrazinolysis followed by separation and identification of the peptide hydrazides. One would expect a simpler mixture of peptide fragments than would be obtained after partial hydrolysis.

Another application of anhydrous hydrazine in protein chemistry concerns its possible use as a solvent. Rees & Singer (1956) found that it dissolved all the proteins tested at room temperature, but there was a continuous decrease with time of the specific viscosity of a sample of γ-globulin in

anhydrous hydrazine at 25°. This is probably due to reaction between the hydrazine and reactive peptide bonds in the protein as shown by the following calculation with glycylglycine. In this case $k_u = 12{\cdot}4 \times 10^{-3}$ min.$^{-1}$ at 60° and, assuming $E = 17$ kcal. mole^{-1}, it is found that, at 20°, 68% of the glycylglycine would have reacted after 50 hr. whereas after the same time at 2° (the freezing point of hydrazine) only 15% would have reacted. The reactivity towards hydrazine of this peptide bond in the protein is probably not very different from that in the dipeptide, hence it may be concluded that, in general, some degradation of proteins will occur even at 2° after prolonged contact with anhydrous hydrazine.

SUMMARY

1. The kinetics of the uncatalysed and acid-catalysed hydrazinolysis of five simple peptides was studied by an experimental technique involving hydrazinolysis, treatment with benzaldehyde, dinitrophenylation and paper chromatography.

2. First-order rate constants were obtained for the uncatalysed reaction and also for the catalysed hydrazinolysis in the presence of a large excess of catalyst. In M-hydrazine sulphate at 60° the reaction is about five times as fast as in anhydrous hydrazine.

3. On the basis of the rate constants it is possible to make recommendations about the use of the hydrazine method for the determination of the *C*-terminal amino acids of proteins.

4. The energies and entropies of activation calculated for two peptides show that the approximately 1000-fold range of rate constants encountered in the catalysed hydrazinolysis at 60° is due largely to steric effects. This may allow selective hydrazinolysis of peptide bonds in proteins.

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The Hydrazinolysis of Insulin, Lysozyme, Wool Proteins and Wool

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Quantitative studies of the hydrazinolysis of proteins have been made by Ohno (1953, 1954, 1955), Locker (1954) and Niu & Fraenkel-Conrat (1955). Ohno (1953) reported yields of up to 68% of leucine from lysozyme which corrected to about 100% after allowance for decomposition of leucine during hydrazinolysis. Locker (1954) obtained only about 25% yields of isoleucine and phenylalanine from tropomyosin and actin respectively, and found that some amino acids were completely decomposed by the hydrazine treatment whereas others could be only partially recovered. Niu & Fraenkel-Conrat (1955) confirmed this observation and, in their work on a number of proteins, corrected for the amount of *C*-terminal amino acid decomposed by the hydrazine (usually more than half). They found that the recovery of amino acids from amino acid plus protein was not appreciably

different from that of the amino acid when treated alone. It is possible to minimize the decomposition of amino acids during hydrazinolysis by working under milder conditions in the presence of an acid catalyst (Bradbury, 1956). This should allow all the amino acids, with the exception of asparagine and glutamine, which are removed in the benzaldehyde treatment, to be determined quantitatively as *C*-terminal in proteins. It was to obtain information on this question, particularly for wool, that this investigation was made.

Preliminary investigations with insulin showed that small amounts of some non-*C*-terminal amino acids were observed in the final solution along with the *C*-terminal amino acid alanine. This probably accounts for the original workers in this field, Akabori, Ohno & Narita (1952), identifying glycine in the hydrazinolysate even although it is not a *C*-terminal residue in insulin. Locker (1954) always found small amounts of non-*C*-terminal amino acids, particularly glutamic acid, serine and alanine, which set a practical limit to the applicability of the method since with high-moleular-weight proteins they become comparable in amount with the quantity of *C*-terminal amino acid. On the other hand, Niu & Fraenkel-Conrat (1955) claim that for the 15 proteins which they investigated, small amounts (0·1 equiv./mole) of non-*C*-terminal amino acids were observed only in a few cases. In a recent paper, Akabori *et al.* (1956) consider the possibility of the formation of these non-*C*-terminal amino acids as a result of hydrolysis of hydrazides during and after hydrazinolysis.

In this paper the acid-catalysed hydrazinolysis method, which is useful for simple peptides (Bradbury, 1956, 1958), is applied to insulin, lysozyme, wool proteins and wool itself. A large number of experiments have been made with the first two in order to study the effect of variation of temperature, water content of the hydrazine and other factors on the yield of *C*-terminal and non-*C*-terminal amino acids. The results obtained with wool show the limitations of the method when it is applied to a protein having very few, if any, *C*-terminal groups.

EXPERIMENTAL

Materials

Samples of bovine insulin (Boots Pure Drug Co. Ltd., Nottingham) and lysozyme (Armour and Co., Chicago) were used without further purification. They were shown to be free of loosely bound amino acids or peptides since after dinitrophenylation no ethyl acetate-extractable dinitrophenyl-(DNP)-amino acids or DNP-peptides were detected on paper chromatograms.

A sample of Merino 64's greasy wool was separated into base and tip and each section extracted in a Soxhlet extractor with light petroleum (Shell X222) for 8 hr. After drying overnight the wool was immersed six times in water, each for 15 min. at 50°, and air-dried at room temperature.

The samples of wool proteins used were kindly supplied by Mr J. M. Gillespie of the Wool Textile Research Laboratory, Melbourne. The material Z29 was the insoluble residue left after successive extractions of wool with alkaline thioglycollate solution. The soluble material from one such extraction was treated with iodoacetic acid to produce *S*-carboxymethylkerateine 2 (SCMK 2) which was fractionated with $(NH_4)_2SO_4$ (Gillespie, 1956, 1957).

Procedure

About 1–2 μmoles (10–100 mg.) of material was weighed into a tube, followed by hydrazine sulphate (0–200 mg.). The material was dried for about 2 hr. at 80° at 10^{-4} mm. Hg pressure and an accurately known volume of hydrazine of known water content (see Bradbury, 1958) was distilled in. The tube was sealed under vacuum, heated and opened, the excess of hydrazine was removed and the residue shaken with benzaldehyde, to remove hydrazides, and dinitrophenylated and extracted as previously described (Bradbury, 1958). The ethyl acetate solution was evaporated to dryness and the bulk of the dinitrophenol removed by two treatments for 30 min. at 40–50° and 10^{-4} mm. Hg pressure, a cold-finger condenser packed with dry ice being used. Test experiments with various DNP-amino acids showed that no appreciable amount of sublimation of these materials occurred under these conditions. The residue was dissolved in acetone and applied as a spot to Whatman no. 3MM paper. The latter is preferred to no. 1 paper because it is more reproducible in behaviour and has a much higher capacity. The paper was run by the ascending method for 16 hr. in the first direction with the toluene solvent and then, after drying at 40° for about 3 hr., in the second direction with the phosphate solvent (Levy, 1954). The spots were marked, those which disappeared with HCl gas were disregarded (including dinitrophenol) and the others tentatively identified by their position with respect to dinitrophenol and a control which was run in the second direction. These spots were cut out, the colour was eluted with 2% $NaHCO_3$ soln., the optical density of the solution measured and the concentration of material in μmoles/g. of protein calculated from, in general, the extinction coefficients in Table 1 of the preceding paper. In most cases there was a brown-yellow streak due to DNP-hydrazides running almost the whole length of the paper in toluene but not moving at all in the second solvent. The aqueous layer from the extraction procedure was not examined, since only mono-DNP-histidine could be present and bis-DNP-histidine was never detected in the two-dimensional chromatogram.

The identity of the spots was checked by hydrolysis with twice-distilled 6N-HCl in a sealed tube at 110° for about 8 hr., and extraction with ethyl acetate. The aqueous layer was evaporated to dryness to remove HCl, and dinitrophenylated and extracted in the usual manner. The DNP-materials from both fractions were then identified by one-dimensional chromatography with the toluene, phosphate or 2-methylbutan-2-ol solvents (Fraenkel-Conrat, Harris & Levy, 1955).

RESULTS

The results are given in Tables 1–3.

DISCUSSION

From Table 1 it is clear that the yield of alanine from insulin is not affected by alteration of the conditions of either the benzaldehyde treatment or the dinitrophenylation, within the limits recorded. The hydrazinolysis reaction is incomplete in the absence of acid catalyst at 60° but complete in M-hydrazine sulphate since no increase of yield occurs on raising the temperature to 80°. As the *C*-terminal sequence of insulin is lysylalanine this result would be predicted from the rate constants in the preceding paper for the uncatalysed and

Table 1. *Hydrazinolysis of insulin and lysozyme*

For definitions of the abbreviations for amino acids used in this table and in Table 3 see *Biochem. J.* (1953), **55**, 5.

		Conditions of hydrazinolysis				Yield of *C*-terminal amino acid* (%)	Non-*C*-terminal amino acids (μmoles/g. of protein)			
Material	Expt. no.	Water in hydrazine (%, w/w)	M-Hydrazine sulphate	Temp.	Time of heating (hr.)		Gly	Ser	Glu	Asp
Insulin	161*b*, 169*a*, 182*a*†	0·0	1·0	60°	16	36±4	10±2	7±1	11±1	0
Insulin	244*a*, 182*b*	0·0	1·0	60	16	37	9	3	10	0
Insulin	161*a*†‡	0·0	1·0	60	16	31	8	13	5	0
Insulin	244*b*§	0·0	1·0	60	16	35	5	4	8	0
Insulin	245	5·0	1·0	60	16	39	6	6	10	0
Insulin	260‖	35·0	1·0	60	16	21	14	9	15	0
Insulin	144‡	0·0	0·0	60	16	16		Not measured		
Insulin	162*b*†	0·0	1·0	80	16	39	11	19	13	0
Insulin	162*a*†‡	0·0	1·0	80	16	34	8	6	14	0
Insulin	145†‡	0·0	0·0	80	16	38	Not measured		13	0
Insulin (1μmole) +alanine (1μmole)	218	0·0	1·0	60	16	46	5	6	10	0
Alanine	201	0·0	1·0	60	16	93	0	0	0	0
Lysozyme	183*a*	0·0	1·0	60	16	77	6	2	0	3
Lysozyme	183*b*‡	0·0	1·0	60	16	54	4	3	0	4
Lysozyme	210	0·0	1·0	60	2	15	1	2	0	1
Lysozyme	195‡	0·0	0·0	60	16	64	0	0	0	v. small
Lysozyme	194*a*	0·0	1·0	80	16	53	14	5	0	6
Lysozyme (1μmole) +leucine (1μmole)	233, 242	0·0	1·0	60	16	82	6	2	0	4
Leucine	243	0·0	1·0	60	16	95	v. small	v. small	0	0

* The *C*-terminal alanine is recovered from insulin (mol.wt. 6000) but not *C*-terminal asparagine, which is removed in the benzaldehyde treatment; the *C*-terminal leucine is obtained from lysozyme (mol.wt. 14 900).

† Dinitrophenylated by Levy's (1954) method; all others by Sanger's (1945) method.

‡ Benzaldehyde treatment with A.R. material in neutral solution. In all other cases treatment was in H_2SO_4 soln. (from N_2H_4,H_2SO_4 after reaction of the hydrazine with benzaldehyde at about pH 2).

§ Treatment with peroxide-free benzaldehyde prepared by double distillation of the A.R. material under reduced pressure of nitrogen (Mulcahy & Watt, 1953).

‖ Insulin had not dissolved completely by the end of the treatment.

Table 2. *Yield of C-terminal amino acids from insulin and lysozyme*

	Insulin*	Lysozyme
Mean yield (% uncorr.)	36±1 (excluding Expts. 260, 144)	62±6 (excluding Expt. 210)
Overall yield (%) of *C*-terminal amino acid from protein plus amino acid added in equimolar amount	46 (Expt. 218)	82 (Expts. 233, 242)
Recovery (%) of the amino acid added to the protein†	56	102
Yield (% corr.)	36×100/56=64	62

* The other *C*-terminal amino acid, asparagine, is converted into the hydrazide and consequently removed by the benzaldehyde treatment.

† This quantity is calculated on the assumption that the yield of, for example, alanine from the insulin in Expt. 218 is 36%, hence the recovery of the alanine added is 2×46−36=56%.

Table 3. *Hydrazinolysis of wool and wool proteins in hydrazine containing* M*-hydrazine sulphate at 60° for 16 hr.*

Protein	No. of run	Yield of amino acids (μmoles/g. of protein)						
		Gly	Ser	Thr	Glu	Asp	Ala	Total
Wool (base)	216, 211	4±2	6±3	3±1	5±1	2±1	4±2	24±10
Wool (base)*	217	3	3	3	2	2	0	13
Wool (tips)	262	3	5	4	2	2	0	16
SCMK 2 (Z34F)	281, 287	0	3±1	0	4±3	3±2	0	10±6
SCMK 2 (Z34F)†	282, 288	7±3	5±2	0	2±2	10±3	0	24±10
Z29	261, 273	0	8	0	6	1	1	15

* No hydrazine sulphate used. † 5% of water in hydrazine.

catalysed hydrazinolysis at 60° of the similar peptide lysylglycine. However, for lysozyme the *C*-terminal leucine is liberated completely at 60° in the absence of catalyst (Expt. 195), hence the *C*-terminal peptide bond must be considerably more labile than that in lysylglycine or leucylglycine (see Fig. 1 of the preceding paper).

Ohno (1955) has proposed the *C*-terminal sequence Asp.Gly.Ala.Asp(NH_2).Leu [for definitions of these abbreviations see *Biochem. J.* (1953), **55**, 5] for lysozyme on the basis of peptides separated after hydrazinolysis, but this result is at variance with a considerable amount of circumstantial evidence which points to Lys.Leu or Arg.Leu (Penasse, Jutisz & Fromageot, 1953; Thaureaux & Acher, 1956; E. O. P. Thompson, private communication). Because of the observed lability of the *C*-terminal peptide bond of lysozyme towards hydrazine a Lys.Leu sequence does not seem likely.

Expts. 201 and 218 show that although alanine itself is not decomposed to any great extent by reaction with hydrazine, about 44% is decomposed in the presence of insulin (Table 2). On the other hand, leucine is not decomposed appreciably by reaction with hydrazine even in the presence of lysozyme. Niu & Fraenkel-Conrat (1955) found no appreciable difference in the recovery of amino acids after uncatalysed hydrazinolysis for 10 hr. at 100° in the presence or absence of proteins, but it must be stressed that their recoveries of alanine and leucine when hydrazinolysed alone were only 45 and 54% respectively. It is not clear why the corrected yield of *C*-terminal amino acid from insulin and lysozyme is only about 60%.

The formation of small amounts of non-*C*-terminal amino acids from insulin and lysozyme must be due to side reactions, the most likely being hydrolysis of hydrazides occurring during hydrazinolysis or in the subsequent treatments or in both (Locker, 1954; Akabori *et al.* 1956). The last-named authors measured the amount of hydrolysis of serine, glycine, alanine and leucine hydrazides after various treatments. They found that serine and glycine hydrazides were hydrolysed, to the extent of about 0·6%, by heating in anhydrous hydrazine (purity > 99%) at 100° for 5 hr., but alanine and leucine hydrazides only to about 0·2%. Dinitrophenylation by Sanger's (1945) method for 2 hr. hydrolysed not more than 0·5% of these hydrazides.

Only a small fraction of the non-*C*-terminal amino acids can be produced by hydrolysis during dinitrophenylation because of the prior removal of the major part of the hydrazides by the benzaldehyde treatment. Akabori *et al.* (1956) suggest that oxidation of hydrazides to acids may be brought about by peroxides present in the benzaldehyde and used *iso*valeraldehyde instead. On comparing Expts. 182*b* and 244*a* with Expt. 244*b* in Table 1, it is clear that the use of twice-redistilled benzaldehyde may cause a slight but not significant lowering of the glycine content over that obtained with A.R. benzaldehyde. The only possible explanation of the formation of most of the non-*C*-terminal amino acids would seem to be that they are produced during hydrazinolysis in anhydrous hydrazine. If this were so one would expect that the amino acids observed should be those which are most readily produced from the hydrazide and, to a lesser extent, those which are most abundant in the protein. Thus the relative abundance of amino acids in insulin is leucine + isoleucine > glutamic acid > valine > glycine, tyrosine > serine, but because of the greater reactivity of the hydrazides of serine and glycine as compared with leucine and alanine (and, by analogy, valine too) the actual order observed is glutamic acid, glycine > serine. With lysozyme the relative abundance of amino acids is aspartic acid > leucine + isoleucine > glycine, arginine, alanine > serine and the non-*C*-terminal amino acids are glycine > aspartic acid, serine.

The obvious way in which the non-*C*-terminal amino acids could be produced from the hydrazides is by hydrolysis, but if so one would expect an increase as the water content of the hydrazine is increased. However, as shown in Table 1 for insulin, there is no change with hydrazine containing 5% of water and only a slight increase even with 35% of water present. Perhaps they are

produced by some reaction other than hydrolysis, but in either event it is seen that it has not been possible to eliminate these non-*C*-terminal amino acids even by the careful exclusion of water from the system by the use of high-vacuum techniques. In the measurement of the *C*-terminal amino acids of other proteins it is therefore necessary to make a correction for the formation of these non-*C*-terminal amino acids.

From the results in Table 3 it is clear that there are no more amino acids produced by hydrazinolysis of the wool proteins SCMK 2 and Z 29 than from wool itself. The alkaline thioglycollate extraction, followed by treatment with iodoacetic acid (for SCMK 2), has therefore produced no new *C*-terminal amino acids, or new *N*-terminal amino acids (Thompson, 1957). Thus the alkaline thioglycollate treatment solubilizes wool by specific rupture of cystine bonds, there being no attack of peptide bonds.

By the use of the hydrazine method on wool, glycine, alanine, serine and threonine were detected qualitatively by Blackburn & Lee (1954). To these must be added glutamic acid and aspartic acid, as shown in Table 3. However, it is very difficult to make a quantitative estimate of the *C*-terminal amino acids because of the occurrence of non-*C*-terminal amino acids and also the losses of *C*-terminal amino acids on hydrazinolysis. The only way to correct for these two factors is to use the data obtained from insulin and lysozyme, although it is realized that this course is open to some objection. It is seen from Tables 1 and 3 that glycine, serine, glutamic acid and aspartic acid from insulin and lysozyme are obtained in slightly greater amount than from wool. Assuming that these are almost entirely non-*C*-terminal in wool leaves only 5 μmoles of *C*-terminal amino acid/g. of protein; about 10 μmoles/g. of protein after allowance is made for the non-quantitative recovery of *C*-terminal amino acids on hydrazinolysis. The *N*-terminal amino acids of wool are the same as those given in Table 3, with valine additional. Their amount has been estimated by various workers with the method of dinitrophenylation and is summarized by Thompson (1957); a total of 16–20 μmoles/g. seems most likely.

SUMMARY

1. The hydrazine method has been used to determine the *C*-terminal amino acids of insulin, lysozyme, wool proteins and wool.

2. The *C*-terminal amino acids, alanine from insulin and leucine from lysozyme, are obtained in 60% yield, together with small amounts of the non-*C*-terminal amino acids, glycine, serine, glutamic acid and aspartic acid.

3. The non-*C*-terminal amino acids appear to be produced during hydrazinolysis by a process which is independent of the amount of water in the hydrazine, at least up to 5%.

4. The total amount of the *C*-terminal amino acids, glycine, serine, threonine, alanine, glutamic acid and aspartic acid, in wool and the wool proteins studied is about 10 μmoles/g.

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The Use of Asparagine and Glutamine for the Biosynthesis of Casein and Plasma Proteins

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The mammary gland is a system in which the components of the blood stream which are used by body tissues for protein synthesis can be readily investigated (Barry, 1956). Previous work has shown that the mammary gland takes the free essential amino acids from the blood stream to provide the essential amino acids of casein (Barry, 1952; Campbell & Work, 1952; Askonas, Campbell, Godin & Work, 1955), and takes free glutamine and glutamic acid to provide the glutamine and glutamic acid residues respectively of casein (Barry, 1956; Sheldon-Peters & Barry, 1956).

Experiments described in this paper show that the mammary gland of the goat takes free asparagine from the blood stream to provide asparagine, but not aspartic acid, residues of casein. They also show that asparagine and glutamine are used in the biosynthesis of plasma proteins to provide asparagine and glutamine residues. These results provide the first evidence for the function of asparagine in animals, and show that the use of glutamine in protein biosynthesis is not confined to casein synthesis.

In this paper 'casein asparagine' refers to the asparagine residues in casein, 'casein aspartic acid' to the aspartic acid residues in casein, and 'casein aspartic acid (acid hydrolysis)' to the aspartic acid in an acid hydrolysate, which is derived from both the casein asparagine and casein aspartic acid.

EXPERIMENTAL

Isotopic compounds

L-[^{14}C]Aspartic acid (uniformly labelled) and D-[^{14}C]-glucose (uniformly labelled) were bought from the Radiochemical Centre, Amersham, and the [^{15}N]ammonium nitrate from the Atomic Energy Research Establishment, Harwell. DL-[1-^{14}C]Glutamine was synthesized as described by Barry (1956).

DL-[^{14}C]*Asparagine* (*uniformly labelled*). This was synthesized by the method of King & Kidd (1951), with L-[^{14}C]aspartic acid (2·8 g.; 50 μc) substituted for their DL-aspartic acid. Paper chromatography showed that the product contained a trace of aspartic acid which was removed by chromatography on Dowex 2 (acetate form). The crystalline product decomposed at 270–280°, after sintering at 210°, and on paper chromatography gave only one spot in the expected position, which showed the delayed colour production with ninhydrin that is typical of asparagine. A sample prepared from unlabelled L-aspartic acid showed no optical rotation in a polarimeter.

Yield (calculated from L-aspartic acid), 27%.

DL-[amide-^{15}N]*Asparagine*. This was made by the same method but with the following modified preparation of phthaloyl-DL-asparagine. $^{15}NH_4NO_3$ (1·5 g.) was dissolved in a little water and excess of saturated KOH solution added. The NH_3 generated was slowly drawn in a stream of air through a drying tube containing KOH pellets and was trapped in a vessel immersed in liquid O_2. Dry ether (40 ml.), previously cooled in liquid O_2, was then added, followed by a solution of phthaloyl-DL-aspartic anhydride (2·3 g.) in dioxan (40 ml.). The phthaloylasparagine was isolated as before.

Yield (per cent of original ^{15}N recovered in DL-[^{15}N]-asparagine), 36%.

Procedure in experiments on goat

The experiments were performed on a goat which was giving about 1 l. of milk per day, and was in good health. Compounds were injected, and blood and milk samples taken, as described by Barry (1952).

Determination of ^{14}C *specific activities*

All compounds were burnt in a stream of O_2 and the specific activity of the CO_2 measured either directly as the gas, or after conversion into $BaCO_3$ (Barry, 1956). The various compounds were isolated as follows.

Asparagine, aspartic acid, glutamine and glutamic acid of casein and plasma proteins. Casein was isolated and hydrolysed by a pancreas suspension as described before (Barry, 1956). This suspension, when incubated alone at 37° for 24 hr., gave no free asparagine or aspartic acid; when incubated with asparagine it produced no aspartic acid, and with aspartic acid it produced no asparagine. The amino acids were separated from the casein digest by dialysis, the diffusate was freeze-dried, and a crystalline sample of aspartic acid was isolated by chromatography on Dowex 1 (Hirs, Moore & Stein, 1954). The fraction containing asparagine was then hydrolysed with HCl and the resulting aspartic acid isolated in the same way.

The plasma proteins, in experiments in which their asparagine and aspartic acid were isolated, were precipitated with 1% (w/v) picric acid by the method of Hamilton & Van Slyke (1943). The precipitate was washed three times with dil. HCl, dissolved in 0·5N-NaOH, neutralized and incubated with the pancreas suspension as before. Picrate ions were removed by passing through a short column of Dowex 2 (chloride form) and aspartic acid and asparagine were isolated as with the digest of casein.

In experiments in which glutamine and glutamic acid of plasma proteins were isolated, the plasma was dialysed overnight against running water to remove the free amino acids, and the proteins were digested in the same way. Glutamic acid, and the glutamic acid produced after acid hydrolysis of glutamine, were isolated on Dowex 1 (Hirs *et al.* 1954).

Aspartic acid (acid hydrolysis) and glutamic acid (acid hydrolysis) of casein and plasma proteins. Casein and plasma proteins were hydrolysed with HCl (Barry, 1952), and crystalline samples of aspartic acid, or glutamic acid, isolated by chromatography on Dowex 1 (Hirs *et al.* 1954).

Free L-asparagine of blood plasma. Since DL-[^{14}C]-asparagine was injected it was necessary to isolate L-asparagine from plasma free from any traces of the D-form. This was done as follows. Blood plasma (50 ml.) was freed from protein by the method of Hamilton & Van Slyke (1943), the excess of picric acid removed with Dowex 2 (Stein & Moore, 1954) and the solution freeze-dried. The residue was dissolved in a little water, brought to pH 4·5, and shaken overnight with glutamic decarboxylase (2 ml.) (Krebs, 1950); this treatment was necessary to remove glutamine, which emerges with asparagine from the columns of Dowex 50 used in the next stage. The supernatant was freeze-dried and eluted from a column of Dowex 50 (100 cm. × 1·15 cm.) as described by Moore & Stein (1951). The fractions containing asparagine (which were proved by paper chromatography to contain no other amino acid) were combined, the asparagine was determined by the ninhydrin method, and a known weight of DL-asparagine added. The solution was then shaken overnight at room temperature with guinea-pig serum (2 ml.) in a dialysis sac. The serum contains an asparaginase (Krebs, 1950) which was found to convert L-asparagine, but not the D-form, into aspartic acid. Salts were removed from the resulting solution (Sheldon-Peters & Barry, 1956) and a crystalline sample of L-aspartic acid was isolated by chromatography on Dowex 1. From the specific activity of this sample the activity of the free L-asparagine of the plasma was calculated, assuming that the D-asparagine of plasma, although possibly detectable by its radioactivity, contributed no significant amount to the asparagine determined by the ninhydrin method. This assumption is justified since the asparagine concentration of plasma was found to be unaffected by the 100 mg. of DL-[^{14}C]asparagine which was injected.

Lactose. This was isolated as described by Reiss & Barry (1953).

Determination of ^{15}N

A known weight of the DL-[^{14}C:*amide*-^{15}N]asparagine used for injection was diluted with a known weight of inactive DL-asparagine and part of this hydrolysed with N-HCl for 2 hr. in the outer chamber of a Conway microdiffusion unit. After evaporation to dryness, saturated KOH solution (2 ml.) was added, and the NH_3 absorbed in 0·25 N-HCl (1 ml.) in the inner chamber. Nitrogen was prepared (Rittenberg, 1946) and the ^{15}N determined in a mass spectrometer. ^{14}C was also determined by combustion of a second sample of asparagine, and the ^{15}N and ^{14}C in the original compound were then calculated.

The amide nitrogen of casein glutamine was isolated and its ^{15}N determined as described by Barry (1956). The amide nitrogen of casein asparagine was isolated as follows. A sample of casein was hydrolysed with the pancreas suspension and the mixture dialysed. The diffusate was brought to pH 7 and heated at 100° for 5 hr., which was found to remove glutamine completely, in agreement with Vickery, Pucher, Clark, Chibnall & Westall (1935). The solution was then subjected to chromatography on a column of Dowex 50 (100 cm. × 1·15 cm.) as described by Moore & Stein (1951), but at room temperature. The fractions containing asparagine were concentrated, and the amide nitrogen was isolated and its ^{15}N concentration determined as described above.

The NH_3 released on acid hydrolysis of casein with 20% (w/v) HCl was also collected in a microdiffusion unit; about 90% is derived from the amide nitrogen of the glutamine and asparagine of casein, and the remainder from the decomposition of other amino acids (Rees, 1946). The remaining nitrogen in the acid hydrolysate was isolated by the Kjeldahl method.

Determination of aspartic acid and asparagine in the blood of guinea pigs

Each guinea pig was killed by decapitation, its blood allowed to drop directly into a weighed amount of 1% (w/v) picric acid, and the weight of blood was found by difference. The free amino acids in the supernatant were separated by chromatography on Dowex 50 exactly as described by Sheldon-Peters & Barry (1956). The fractions containing a mixture of glutamine and asparagine were heated at 100° for 3 hr. with conc. HCl (4 ml.), and the resulting aspartic acid was determined after a second elution from the column. The fractions which contained a mixture of aspartic acid and threonine were desalted (Sheldon-Peters & Barry, 1956), and eluted from the column as before, but with the buffer at pH 4·25; the aspartic acid emerged in a single peak and was determined.

RESULTS

Goat's milk contains about 3% of casein, in which the amino acid concentrations are very similar to those in cow's casein (Davis & Macdonald, 1953). On acid hydrolysis, 100 g. of cow's casein yields 7·1 g. of aspartic acid, which is derived from both the asparagine and aspartic acid residues, the relative proportions of which are not known (Gordon, Semmett, Cable & Morris, 1949). Goat's blood plasma contains about 0·95 mg. of free asparagine/100 ml. (Barry, 1953), and about 0·1–0·2 mg. of free aspartic acid. The asparagine and aspartic acid of casein might therefore be derived from the free asparagine or aspartic acid of plasma, or from plasma proteins, or might be synthesized within the gland from other compounds absorbed from the blood.

The first experiment (Fig. 1) was designed to find whether either the asparagine or aspartic acid of casein is derived from the free asparagine of blood. [^{14}C]Asparagine was injected intravenously into a goat, thus labelling the free asparagine of its blood, and blood and milk samples were taken at intervals after the injection. It can be seen that the specific activity of asparagine in each casein sample

is many times that of aspartic acid, showing that they have different origins. The activity of asparagine in the first sample is over 40 times that of aspartic acid; it is also over 40 times the activity of aspartic acid (acid hydrolysis) from the plasma proteins at 0·33 and 6 hr. It is also seen that the specific activity of the free asparagine of blood at 0·33 hr. is about three times the highest value for casein asparagine, and that the activity at 6 hr. is somewhat lower than the lowest casein asparagine value. From the closeness of their specific activities it is clear that the free asparagine of blood is an important precursor of casein asparagine. If the asparagine residues of casein are derived solely from the free asparagine of blood, then the area under the casein asparagine histogram will equal the area under a curve showing the specific activity of the free asparagine of blood up to 6 hr. (Barry, 1956). Because of the technical difficulty, the specific activity of free L-asparagine was measured in only two blood samples, and a curve cannot be plotted. However, it can be predicted from previous similar experiments in which complete specific activity curves for other free amino acids were obtained with points intermediate between 0·33 and 6 hr., that if the curve were completed the area under it would be approximately equal to that beneath the casein asparagine histogram. It may therefore be concluded that the asparagine residues of casein are largely derived from free asparagine taken from the blood stream by the mammary gland; since a complete curve for free asparagine is lacking, it cannot be concluded with certainty, in the way it could before (Barry, 1952, 1956), that there is no other quantitatively important source of casein asparagine residues. The possibility that free asparagine of blood is converted into a derivative, such as a peptide, before being absorbed by the mammary gland may be excluded for reasons similar to those mentioned before (Barry, 1952, 1956).

A further experiment (Table 1) shows that the free asparagine of blood does not exchange its amide nitrogen when it is incorporated into casein. The asparagine of blood was labelled by injecting [^{14}C:*amide*-^{15}N]asparagine, and casein was isolated from a milk sample taken 3 hr. after the injection. It can be seen that the concentration of ^{15}N in the

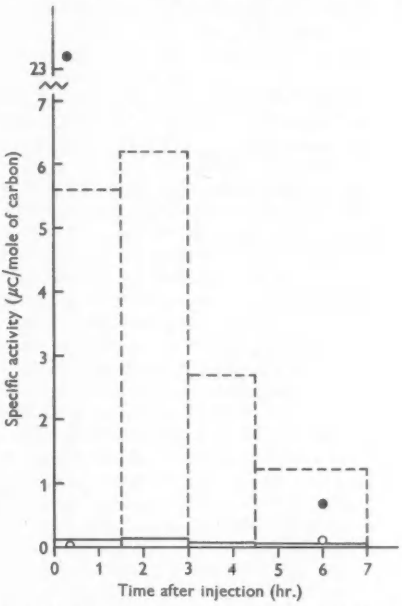

Fig. 1. Use of asparagine for synthesis of casein. DL-[^{14}C]Asparagine (100 mg.; 1·76 μc) was injected intravenously into a lactating goat. The goat was milked immediately before injection, and blood and milk samples were taken at intervals up to 7 hr. after. ●, Free L-asparagine of blood plasma: ○, aspartic acid (acid hydrolysis) of plasma proteins. The histograms show the specific activities of casein asparagine (broken lines) and casein aspartic acid (solid lines) in each milk sample, the width of each rectangle occupying the time during which the milk sample was secreted.

Table 1. *Incorporation of doubly labelled asparagine into casein*

DL-[^{14}C:*amide*-^{15}N]Asparagine (360 mg.; 2·98 μc) injected intravenously into goat and milk taken 3 hr. later.

	Atoms % excess of ^{15}N	Specific activity (μc/mole of carbon)	μc/mole of carbon / Atoms % excess of ^{15}N
Injected asparagine	26·8 (*a*)	273	10·2
Casein asparagine	0·750 (*a*)	8·57	11·4
Casein aspartic acid	—	0·808	—
Casein aspartic acid (acid hydrolysis)	—	5·44	—
Casein glutamine	0·167 (*a*)	—	—
Amide N casein (*b*)	0·418	—	—
Non-amide N of casein (*c*)	0·004	—	—

(*a*) Figures are for amide nitrogen. (*b*) Nitrogen released as NH_3 on hydrolysis with 20% (w/v) HCl. (*c*) Nitrogen not released as NH_3 on hydrolysis with 20% (w/v) HCl.

amide nitrogen of casein asparagine is nearly 200 times that in the non-amide nitrogen of casein, and that the ratio $^{14}C:^{15}N$ is approximately the same in the casein asparagine as in the injected asparagine. From the specific activities of asparagine, aspartic acid and aspartic acid (acid hydrolysis) in Table 1 it can be calculated that 59·8 % of the aspartic acid in the acid hydrolysate of casein came from asparagine residues. This figure may not be exact since it may have accumulated the error in each of the three specific activities, but it suggests that if 100 g. of casein was hydrolysed without decomposition of asparagine it would yield 4·3 g. of asparagine and 2·8 g. of aspartic acid.

A further experiment (Fig. 2) shows that there was little synthesis of casein asparagine or aspartic acid from blood glucose within the mammary gland. It has been demonstrated previously that blood glucose is the principal compound used for lactose synthesis by the mammary gland of the goat (Reiss & Barry, 1953). Therefore, after injection of [^{14}C]glucose, the area under the specific activity–time histogram of a compound in milk which is synthesized solely from blood glucose must be at least as large as the area under the lactose histogram (Barry, 1956). In Fig. 2 the area under the casein aspartic acid (acid hydrolysis) histogram is only about one-sixth of that under the lactose histogram; therefore not more than one-sixth of the casein aspartic acid (acid hydrolysis) can have been synthesized in the mammary gland from glucose. In the first casein sample from this experiment the specific activities of asparagine, aspartic acid and aspartic acid (acid hydrolysis) were 3·62, 7·27 and 5·95 μc/mole of carbon respectively, which again shows that casein asparagine and aspartic acid have different origins. It may be concluded that not more than one-sixth of the asparagine residues of casein were synthesized within the mammary gland from blood glucose; in fact the figure is probably less than one-sixth, the activity of casein asparagine being at least partly due to the synthesis of blood asparagine from the injected glucose outside the mammary gland.

Fig. 2. Synthesis of lactose and casein aspartic acid (acid hydrolysis) from blood glucose. D-[^{14}C]Glucose (uniformly labelled) (224 mg.; 71 μc) was injected intravenously into a lactating goat. The goat was milked immediately before injection and at intervals up to 7 hr. after. The histograms, plotted as in Fig. 1, show the specific activities of lactose (broken lines) and casein aspartic acid (acid hydrolysis) (solid lines) in each milk sample.

The results of this and our earlier work show that the free asparagine and glutamine of blood plasma are used, in the biosynthesis of casein, to provide asparagine and glutamine residues, but no significant portion of the aspartic or glutamic acid residues. The data in Tables 2 and 3 show that the free asparagine and glutamine of blood are used in the same way in the biosynthesis of plasma proteins. The results in Table 2 show that after labelling the free asparagine of the goat's blood with [^{14}C]asparagine, the asparagine of plasma proteins gradually became radioactive, the specific activity in each sample being about ten times that of the aspartic acid. (The possibility that the labelling of plasma proteins is due to physical adsorption of asparagine is excluded by the fact that a plasma-protein sample taken 20 min. after injection, when the activity of the free asparagine was highest, had negligible activity.) Similarly, the results in Table 3 show that after the free glutamine of plasma was labelled the glutamine residues of plasma proteins gradually became active, and that their activity was always about seven times that of the glutamic acid residues.

We have shown that asparagine is used in protein biosynthesis in the goat, and therefore probably also in other animals, such as man and the cat, whose blood plasma contains similar concentrations of free asparagine (Stein & Moore, 1954; Tallan, Moore & Stein, 1954). The blood plasma of guinea pigs, unlike that of other animals which have been studied, contains an asparaginase (Krebs, 1950), and we were interested to see whether it also contained asparagine. Three guinea pigs were killed by decapitation, the blood of each was allowed to drop directly into picric acid solution, and asparagine and aspartic acid were determined in each protein-free supernatant. The concentrations were too low to be accurately measured, but each animal had about 0·1 mg. of asparagine/100 ml. of whole blood, and about 0·3 mg. of

Table 2. *Incorporation of labelled asparagine into plasma proteins*

Specific activities of amino acids in plasma-protein samples taken at intervals after injecting DL-[^{14}C]asparagine (uniformly labelled) (250 mg.; 4·4 μc) intravenously into goat.

Time of sampling (hr.)		0·33	1·5	4·0	6·0
Specific activity of total carbon (μmc/mole of carbon)	Asparagine	—	154	351	475
	Aspartic acid	—	20	29	33
	Aspartic acid (acid hydrolysis)	<20	93	211	276

Table 3. *Incorporation of labelled glutamine into plasma proteins*

Specific activities of amino acids in plasma-protein samples taken at intervals after injecting DL-[1-^{14}C]glutamine (101 mg.; 9·04 μc) intravenously into goat.

Time of sampling (hr.)		0·33	1·5	2·5	4·0
Specific activity of total carbon (μmc/mole of carbon)	Glutamine	—	92	101	132
	Glutamic acid	—	12	19	22
	Glutamic acid (acid hydrolysis)	<10	53	74	97

aspartic acid/100 ml. The asparagine concentration is thus about one-tenth of that in the blood of man, the cat, the goat (Stein & Moore, 1954; Tallan *et al.* 1954; Barry, 1953) and the cow (unpublished work).

The blood of guinea pigs was also found to contain a compound which reacts with ninhydrin, and which is not found in the blood of man, the goat or the cow. It was eluted from the columns of Dowex 50 by the buffer of pH 3·42 together with threonine and aspartic acid, but emerged before them with buffer of pH 4·25. It gave a blue ninhydrin colour, equivalent to about 0·6 mg. of aspartic acid/100 ml. of whole blood, but was not identified.

DISCUSSION

We have shown that the mammary gland takes free asparagine from the blood stream to provide asparagine, but not aspartic acid, residues of casein, and that free asparagine also provides asparagine residues of plasma proteins. The compound or compounds that are taken from the blood stream by the mammary gland to provide the aspartic acid residues of casein have not yet been discovered. Asparagine has been found in animal tissues by Krebs (1950) and also occurs in urine and blood plasma (Ågren & Nilsson, 1949; Stein, 1953; Stein & Moore, 1954; Barry, 1953), but there has previously been no evidence for its function. Waelsch (1952) tentatively suggested that β-aspartyl peptides formed from asparagine are intermediates in protein synthesis. Our results (Fig. 1, Tables 1 and 2) suggest that asparagine does not form these intermediate peptides when it is incorporated into casein and plasma proteins, for reasons similar to those mentioned with reference to glutamine (Barry, 1956).

The evidence that no significant part of the casein asparagine was synthesized from blood glucose is consistent with the conclusion that casein asparagine comes directly from the blood, for, if it had been synthesized within the gland, the glucose, lactate or propionate of the blood would probably have been used to make it. [Mammals apparently cannot use acetic acid for the net synthesis of C_4 compounds, although Kornberg & Krebs (1957) suggest that animals on fatty diets may, like bacteria, become adapted to do this. It is conceivable that ruminants become adapted since their metabolism is in many ways like that of animals fed on large amounts of fat. The difficulty that propionate is the only product of digestion which is known to be able to provide any of the large carbohydrate output of a lactating ruminant would then disappear.]

The results of this and previous work (Barry, 1956) show that free glutamine provides the glutamine residues, but no significant portion of the glutamic acid residues, of both casein and plasma proteins. It is clear that the glutamine, glutamic acid, asparagine and aspartic acid of casein and plasma proteins must be considered as four independent amino acids which are not interconverted during or after their incorporation into the protein. Glutamine and asparagine residues occur in most animal and plant proteins, and it seems likely that they are in general derived from free glutamine and asparagine of the tissues. This idea is supported by the following evidence that cells in tissue culture use the free glutamine of their medium to provide the glutamine residues of new protein. Rabinovitz, Olson & Greenberg (1956) found that, if glutamine formation by Ehrlich ascites cells was inhibited, the incorporation of other amino acids into protein was also inhibited; and if labelled glutamine was added to the medium of growing cells, its carbon was incorporated into protein. Also, human uterine carcinoma cells of the strain HeLa, when in tissue

culture, need glutamine for growth. Levintow (1957) labelled the culture medium of these cells with [^{14}C:*amide*-^{15}N]glutamine, and showed that the glutamine residues of new protein were derived from the medium without change in the ^{14}C:^{15}N ratio.

It seems therefore that glutamine and glutamic acid, and asparagine and aspartic acid, should be considered as distinct from one another as are any other non-essential amino acids. It is true that the free glutamine of blood is continually synthesized from glutamic acid (Barry, 1956), but many other non-essential amino acids, such as serine and glycine, are also rapidly interconverted. This distinction may have been overlooked, because of the conversion of asparagine and glutamine into aspartic and glutamic acids which occurs when proteins are hydrolysed with acid, particularly in interpreting experiments of two types:

(i) The first type is that in which conclusions are drawn from the concentrations of isotope in glutamic and aspartic acid in acid hydrolysates of protein. For example, in the experiment of Steinberg & Anfinsen (1952), crystalline ovalbumin, isolated from hen oviducts which had been incubated with $^{14}CO_2$, was partially hydrolysed enzymically, and the resulting fractions of low and high molecular weight were separated and hydrolysed with acid. The specific activities of glutamic and aspartic acid in one hydrolysate were found to differ from those in the other, and it was concluded that both the glutamic and aspartic acid residues of ovalbumin had different activities in different parts of the molecule. This conclusion would be justified only if glutamic and aspartic acid had been isolated after enzymic digestion of the two fractions under conditions in which glutamine and asparagine are not hydrolysed, since the results could equally well be explained by the amides and acids having different specific activities, and being in different proportions in the two fractions. (These authors have, however, unequivocal evidence that the alanine residues of ovalbumin, isolated after incubating oviducts with [^{14}C]alanine, do not have identical activities.)

(ii) Experiments of the second type are those in which requirements of cells for glutamine or asparagine have been found (see review by Waelsch, 1952). The possibility that these are normal amino acid requirements for protein synthesis has rarely been considered, and much energy has often been spent, with little success, in attempting to find special functions of glutamine and asparagine in metabolism. These functions certainly exist, examples being the use of glutamine in purine biosynthesis (Hartman, Levenberg & Buchanan, 1956), and glucosamine biosynthesis (Leloir & Cardini, 1953), but the fact that asparagine and glutamine are protein amino acids should be remembered. As mentioned above, this fact has recently been found to explain the earlier discovery of a requirement of cells in tissue culture for glutamine.

SUMMARY

1. From experiments with asparagine labelled with ^{14}C and ^{15}N it is concluded that the lactating mammary gland of the goat takes free asparagine from the blood stream to provide asparagine, but not aspartic acid, residues of casein.

2. This conclusion agrees with the finding that there is little synthesis of casein asparagine from glucose within the mammary gland.

3. It is also shown that asparagine and glutamine are used in the biosynthesis of plasma proteins, to provide asparagine and glutamine residues.

4. It is clear that glutamine and glutamic acid, and asparagine and aspartic acid, are not interconverted during or after their incorporation into casein or plasma proteins. It seems therefore that they should be considered as four distinct non-essential amino acids.

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Effect of pH on the Activity of Eel Esterase towards Different Substrates

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Much fundamental information about the structure of the active centre in cholinesterases has been derived from studies on changes of activity with pH. These enzymes show a maximum activity at about pH 7–8. On the acid side, activity falls off in approximately the same manner for all substrates studied. However, in the alkaline range great differences were observed between different esters (Bergmann, Segal, Shimoni & Wurzel, 1956). The bell-shaped form of the pH–activity curves has been interpreted to indicate two different components of the esteratic site, one of which (the nucleophilic group G_1) is inactivated competitively by H^+ ions and the other (the electrophilic group G_2) by OH^- ions. Such an interpretation permits the calculation of the respective dissociation constants, pK_a and pK_b. The determination of the dissociation constants, in turn, leads to some speculation about the possible nature of these two groups (Bergmann *et al.* 1956).

A new argument about the influence of pH on esterase activity has been raised recently by Kistiakowsky & Mangelsdorf (1956). These authors observed that hydrolysis of methyl butyrate by horse-liver esterase varies according to reaction conditions. If reaction rates were measured immediately after addition of the enzyme to the alkaline buffer and for short periods, no decrease in rate in the alkaline range (beyond pH 9) was found. If, however, a pre-incubation period of about 20–60 min. preceded the addition of substrate to the enzyme–buffer mixture, the usual decrease in activity with increasing pH became apparent. The authors concluded from their results that the 'true' activity does not change, but that the enzyme protein undergoes slow changes in the alkaline range, which at first are reversible but progressively become irreversible. These changes are assumed to simulate the competition of substrate and OH^- ion for the active surface, which forms the basis of the above-mentioned interpretation of the pH–activity curve.

In the following experiments we have tried to elucidate this problem and to test our previous assumptions about the role of the electrophilic group G_2 in the hydrolytic mechanism.

MATERIALS AND METHODS

Substrates. Phenyl acetate, *p*-methoxyphenyl acetate and *p*-nitrophenyl acetate were prepared by refluxing the appropriate phenol with excess of acetic anhydride and subsequent distillation. Standard solutions of these esters were prepared in water, except for *p*-nitrophenyl acetate, which shows appreciable hydrolysis in aqueous solution. Therefore an M-solution in dioxan was diluted 1:2500 with water, immediately before use, to give a final concentration of 0·4 mM. The dioxan present was found to be devoid of any inhibitory effect in such dilutions.

Acetylthiocholine (AThCh) was the commercial product of Hoffmann-LaRoche, Basel, Switzerland.

Enzyme. Eel esterase was prepared from the electric organ of *Electrophorus electricus* according to the method of Nachmansohn & Rothenberg (1945). The standard preparation, diluted 1:7500, with 4 mM-acetylcholine (ACh) as substrate, hydrolysed 8 μmoles/hr./ml. at 37°.

Measurement of activity. The following buffers were used to cover the pH range required: 0·1 M-sodium acetate for pH 5·0–6·5; 0·1 M-sodium phosphate for pH 6·5–8; 0·1 M-sodium pyrophosphate for pH 8·0–9·5; aq. NH_3 soln.–ammonium acetate for pH values above 9·5.

For manometric measurements, a buffer of the following composition was used: NaCl, 0·1 M; $NaHCO_3$, 0·025 M; $MgCl_2$, 0·04 M. The pH was adjusted to 7·4. The gas phase was CO_2 + air (5:95, v/v).

Hydrolysis of AThCh was measured either by the Warburg manometric method (Ammon, 1933) or colorimetrically under conditions which are suitable for the determination of ACh (Hestrin, 1949). Non-enzymic hydrolysis of AThCh is negligible up to pH 10. Therefore this ester is the substrate of choice for the standardization of cholinesterase preparations. The pS–activity curve of AThCh, shown in Fig. 1, is different from that of ACh, although the maximum appears in the same concentration range

Hydrolysis of the phenyl acetates was measured spectrophotometrically, use being made of the large difference in $\lambda_{max.}$ between phenols and their acetates. As an example, Fig. 2 shows the ultraviolet-absorption spectra of *p*-nitrophenol, the corresponding phenoxide and the acetate. In order to make the calculation of rates at various pH values independent of the actual ratio phenol:phenoxide, the isosbestic point at 348 mμ was selected, although the sensitivity of the method is thus less than at the absorption maxima (320 and 400 mμ respectively). The relevant spectroscopic data for the three phenyl acetates used in this investigation are summarized in Table 1. As can be seen, for phenyl acetate and its *p*-methoxy derivative, which were used throughout at mM concentration, the extinction (E) of the ester is zero at the isosbestic point of phenol and phenoxide. The situation is somewhat different for the *p*-nitro series. Here, the acetate, which was used in a concentration of 0·4 mM, has an extinction of 0·152 at 348 mμ. Therefore the progress of hydrolysis in the time interval $t_2 - t_1$ is given by the formula

$$x_2 - x_1 = \frac{E_{t(2)} - E_{t(1)}}{E_2 - E_1},$$

where x designates the concentration of phenol, E_t the actual extinction readings and E_2 the extinction of 0·4 mM-*p*-nitrophenol at 348 mμ, whereas E_1 indicates the extinction of the corresponding acetate at the same wavelength.

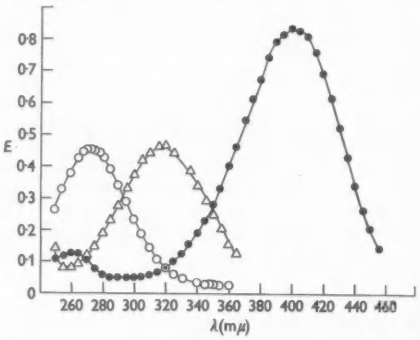


Fig. 2. Ultraviolet-absorption spectra. ●, *p*-Nitrophenoxide ion; △, *p*-nitrophenol; ○, *p*-nitrophenyl acetate. Concentrations, 0·05 mM.

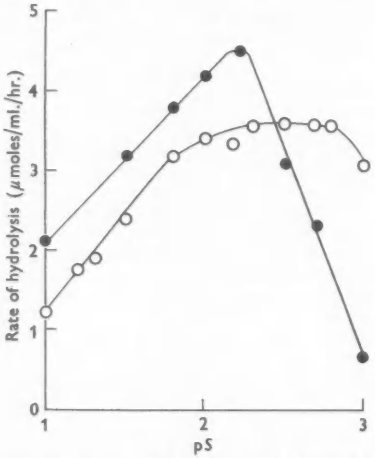


Fig. 1. pS–activity curves of eel esterase. Enzyme dilution 1:15 000, pH 7·4; temperature 37°. Rates of hydrolysis were measured by the Warburg manometric method. ●, Acetylcholine; ○, acetylthiocholine.

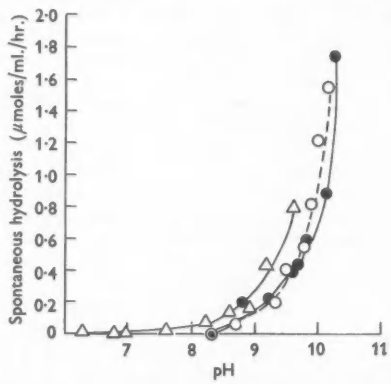


Fig. 3. Non-enzymic hydrolysis of phenyl acetates as a function of pH. △, *p*-Nitrophenyl acetate, 0·4 mM; ○, *p*-methoxyphenyl acetate, mM; ●, phenyl acetate, mM.

Table 1. *Spectroscopic data on phenols and their acetates*

Substituent	$\lambda_{max.}$ of Ester	$\lambda_{max.}$ of Phenol	$\lambda_{max.}$ of Phenoxide	Isosbestic point of phenol and phenoxide	E_2*	E_1*	pK of phenol
H	220	270	288	272·5	1·36	0	9·9†
p-OCH_3	275	288	308	295	2·12	0	10·2†
p-NO_2	271	320	400	348	5·20	0·38	7·0‡

* Extinction of mM solutions. E_2, Extinction of phenol or phenoxide at their isosbestic points; E_1, extinction of the ester at the isosbestic point of phenol and phenoxide.

† Data of Fletcher (1946).

‡ Data of Clark (1928).

For evaluation of rates, only the initial linear part of the curve, representing E_t as a function of time, was used, i.e. that part where $x_2 - x_1$ is constant for a given time unit.

All measurements were carried out with a Beckman ultraviolet spectrophotometer, the temperature of 27–28° being maintained with the aid of a Beckman thermospacer.

Enzymic minus spontaneous hydrolysis was expressed as percentage of the maximum enzymic rate. The relative rates so obtained were then plotted as functions of pH, for comparative purposes. The pH dependence of the spontaneous hydrolysis of phenyl acetate and its *p*-nitro and *p*-methoxy derivatives is shown in Fig. 3.

RESULTS

Hydrolysis of acetylthiocholine by true cholinesterase as a function of pH

In Fig. 4 the pH–activity curve for the system eel esterase–AThCh is compared with that of ACh. All points above pH 8 were measured twice, (*a*) by adding the enzyme to the substrate–buffer mixture and taking readings at once, i.e. after incubation for only 0·5 min., and (*b*) by incubating enzyme and buffer for 20 min. before addition of the substrate. In each case identical results were obtained under these two conditions, so that the length of the incubation period had no effect on the enzymic rates. Only at the highest pH studied (10·5), when incubation was extended beyond 1 hr., was a decrease in activity observed under condition (*b*). Even such drastic treatment produced only a reversible change in the enzyme protein, since after readjustment of the pH to 9·5 the original activity was recovered. The results in Fig. 4 therefore are reliable evidence that AThCh, in contrast with ACh and all other *O*-alkyl esters studied previously (Bergmann, 1955), shows no decrease in rate of enzymic hydrolysis up to pH 10·6. This behaviour would be consistent with the assumption that a certain group in the enzyme, which forms a link with *O*-alkyl esters, cannot attach itself to the thiol ester. The most pronounced difference between the two atoms involved lies in their ability to form hydrogen bonds. The results of Fig. 4 thus suggest that a group is present in the active surface of eel esterase, which forms a hydrogen bond to the ethereal oxygen atom (i.e. attached to the alkyl radical) of the ester group in ACh and loses this ability progressively at pH values above 8.

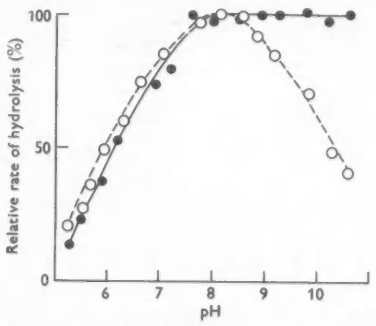
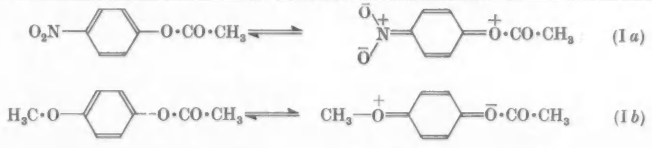


Fig. 4. Hydrolysis of choline esters by eel esterase as a function of pH. Enzyme, 1:1500; temp. 27°. ○, Acetylcholine, 5 mM; ●, acetylthiocholine, 6 mM.

Rates of enzymic hydrolysis of phenyl acetates at different pH *values*

In order to test this hypothesis, a group of esters has been taken in which the structure of the alcoholic portion would produce an effective change of the electron density at the ethereal oxygen atom and thus alter its hydrogen-bonding capacity. The benzene ring in phenyl acetate can function as an 'electron sink'. Furthermore, it would be expected that a *p*-nitro group, by placing a formal positive charge on the ethereal oxygen atom, as in the reaction sequence (I*a*), would enhance the general effect of the benzene ring. On the other hand, a *p*-methoxy substituent (I*b*) will produce a formal negative charge on the ethereal oxygen atom and thus diminish the effect of the aromatic ring. The phenyl acetates used have been tested previously as substrates of erythrocyte cholinesterase (Mounter & Whittaker, 1953). However, the influence of pH on their rates of hydrolysis has not been investigated. The pH–activity curves of these esters were measured both after 1 and 20 min. incubation, but no difference was found up to pH 10·5. The curves in Fig. 5 show that over the range from pH 7 to 9·5 the rates remain constant. Unfortunately, with *p*-nitrophenyl acetate the measurements could not be extended beyond this range, in view of the fast non-enzymic hydrolysis of this ester. With the other two esters, a rapid decrease of activity appears between pH 9·5 and 10·5. The question whether the *p*-nitro group delays the decreased rate of enzymic activity in the alkaline range has not been answered by these experiments.



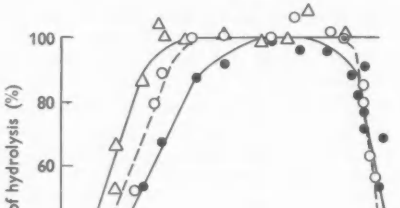


Fig. 5. pH–activity curves for the system eel esterase–phenyl acetates. Hydrolytic rates at 28° were determined by the spectrophotometric method. The maximum rates are designated as 100%. With *p*-nitrophenyl acetate, the plateau at 100% relative rate was calculated from the average value of eight experimental points covering the range pH 6·5–9·5. Therefore the points lying above or below this plateau express the experimental error of the method used. △, *p*-Nitrophenyl acetate; ○, *p*-methoxyphenyl acetate; ●, phenyl acetate; concentrations as in Fig. 3.

Dependence upon pH *of imidazole-catalysed hydrolysis of esters*

It has long been suspected that the imidazole ring of histidine represents the nucleophilic group G_1 of the esteratic site (Wilson & Bergmann, 1950). The catalytic effect of imidazole and some of its derivatives has been demonstrated for di*iso*propyl phosphorofluoridate (DFP) hydrolysis by Wagner-Jauregg & Hackley (1953) and for phenyl acetates by Bruice & Schmir (1956). Esters of aliphatic alcohols are not attacked.

The dependence of imidazole-catalysed hydrolysis upon pH has been determined recently by Bruice & Schmir (1957) in the range pH 6·3–7·9. We have extended these measurements as far as possible into the alkaline range to show that above pH 7 the reaction becomes independent of pH (Fig. 6).

The pH–activity curve of the system eel esterase–AThCh in Fig. 4 suggests that this ester can be split by interaction with G_1 only, without interference of G_2. If this explanation is correct, then imidazole alone should be able to split esters of the general structure R·CO·S·alkyl, although it is ineffective towards the corresponding *O*-alkyl esters. We have indeed found that AThCh is split by imidazole at about the same rate as *p*-methoxyphenyl acetate (Table 2).

Fig. 6. Dependence upon pH of imidazole-catalysed hydrolysis of *p*-nitrophenyl acetate. Imidazole, mM; ester, 0·4 mM; temp. 28°. Concentration of dioxan in the reaction mixture, 5%. Hydrolysis was measured by the spectrophotometric method. The constant rate, observed between pH 7·5 and 9·5, is used as reference (=100%). All other rates were expressed as percentages of this maximum rate.

Table 2. *Effect of imidazole on the rate of hydrolysis of* p*-methoxyphenyl acetate and acetylthiocholine*

Hydrolysis was measured by the Warburg manometric method, at pH 7·5 and 37°. Concentration of both imidazole and ester, 10 mM. The figures in col. 2 represent the difference between total and spontaneous rates.

Substrate	Rate of catalysed reaction (μmole/ml./hr.)	Spontaneous rate (μmole/ml./hr.)
p-Methoxyphenyl acetate	0·85	0·36
Acetylthiocholine	0·73	0·17

DISCUSSION

The experiments reported here demonstrate that eel esterase equilibrates with the OH^- ions of the medium so fast that the length of the incubation period has no influence on hydrolytic rates. The shape of the pH–activity curves, measured with eel esterase in the alkaline range, thus represents rapid reversible changes taking place in the active surface. It may well be that the different observations of Kistiakowsky & Mangelsdorf (1956) are due to a peculiar behaviour of their enzyme, which apparently equilibrates rather slowly with OH^- ions. Their findings in no way invalidate conclusions, drawn from the shape of pH–activity curves, about the structure of the active centres.

The dependence on pH in the system eel esterase–substrate varies markedly in the alkaline range,

according to the structure of the ester. Acetylthiocholine hydrolysis remains constant above pH 7, in sharp contrast with *O*-alkyl esters. This fact affords strong evidence that the decrease in reaction rate, observed with *O*-alkyl esters in the alkaline range, is real and leads to the conclusion that the active centre of the enzyme studied includes a group which (*a*) forms a hydrogen bond to the ethereal oxygen of the ester group, and (*b*) loses its catalytic activity with increasing OH^- ion concentration. This group is thus identical with the previously assumed 'electrophilic group G_2'. The greater the affinity of a substrate for this group G_2, the more pronounced is the enhancement of enzymic rate produced by G_2, and consequently the more rapid is the decrease in rate of enzymic hydrolysis at higher pH values. The pK values, derived previously for this component of the active surface, indicated as one possibility that the phenolic hydroxyl of tyrosine may be involved (Bergmann *et al.* 1956). Recently, it was found by Holland & Klein (1956) that diazonium compounds inactivate erythrocyte cholinesterase. This result again led to the assumption that a tyrosine unit participates in the active centre.

G_2 clearly exerts its effect, before the alcoholic component of the ester is split off, since, as seen in reaction sequence (II), the intermediate acylenzyme is identical for ACh, AThCh and the three phenyl acetates. Therefore the large differences in the hydrolytic rates of these five substrates, summarized in Table 3, must be due to differences in the velocity constants, characteristic for step (1) or (2) or both. This proves at the same time that step (3) cannot be the rate-determining step, at least not for acetates. G_2 has no effect on the hydrolysis of AThCh up to pH 10·6. Which role then does it play in the splitting of *O*-alkyl esters? In the acid-catalysed hydrolysis, the C–O–alkyl bond becomes polarized by attachment of a proton (Ingold, 1953). In a similar fashion, formation of a hydrogen bond polarizes the *O*–alkyl bond and thus facilitates both the removal of R′·OH and the nucleophilic attack of G_1 on the carbonyl carbon atom. Such an effect is missing or unimportant in the formation and splitting of the complex thiol ester–cholinesterase.

We have also observed that thiono esters, R·CS·O·alkyl, are not attacked at all by esterases (F. Bergmann & R. Segal, unpublished work). In all imidazole-catalysed hydrolyses, the nucleophilic nitrogen, which is similar to the OH^- ion, combines with the ester group to form the transition state (III). A negative charge is placed on the carbonyl oxygen atom, owing to the formation of the ortho ester-like intermediate. Such a charge distribution is still possible in the thiol esters, but cannot materialize in the thiono esters, because the electronegativity of sulphur is of the same magnitude as that of carbon (Pauling, 1948).

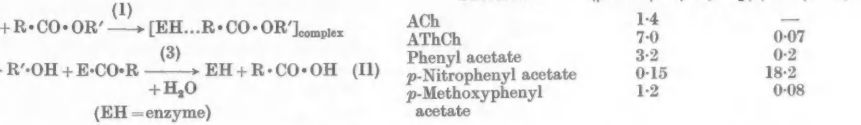
These considerations on the substrate specificity of eel esterase may be summarized as follows: (1) In a thiol ester the imidazole nitrogen attacks the carbonyl carbon atom, and produces an ortho ester-like intermediate. Here the intrinsic polarizability of sulphur is sufficient to break the C–S–alkyl bond. (2) In an *O*-alkyl ester, the ortho ester structure as such is stable and requires additional 'activation' by hydrogen bonding. (3) In a thiono ester the double-bonded sulphur atom is unable to acquire a negative charge. Therefore combination with the imidazole ring is impossible and hydrolysis does not take place.

These conclusions are corroborated by analogous findings in the phosphate series. Thus it has been found by Diggle & Gage (1951) that the thiol

Table 3. *Comparison of rates of hydrolysis of phenyl acetates*

All determinations were carried out at pH 7·5 and 28°. With eel esterase, all substrates were studied at mM-concentration and the rates calculated for an enzyme dilution of 1:7500, a linear dependence on enzyme concentration being assumed. In the imidazole-catalysed reactions, k_2 represents the bimolecular velocity constant, based on the equation for initial rates: $v_2 = k_2$[imidazole] × [ester].

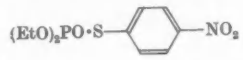
Substrate	Rate of hydrolysis by eel esterase (μmoles/ml./hr.)	Imidazole catalysis k_2(l./mole/min.)
ACh	1·4	—
AThCh	7·0	0·07
Phenyl acetate	3·2	0·2
p-Nitrophenyl acetate	0·15	18·2
p-Methoxyphenyl acetate	1·2	0·08



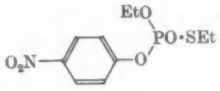
(II)

(III)

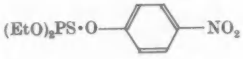
phosphates (IV) and (V) are active inhibitors of cholinesterases, whereas the thionophosphate (VI), i.e. pure parathion, is inactive.



(IV)



(V)



(VI)

In this series of esters, too, a substrate containing a double-bonded sulphur atom cannot be split by cholinesterases.

It is remarkable that the rates of enzymic hydrolysis of phenyl acetates do not follow the same order as the imidazole-catalysed reaction. Mounter & Whittaker (1953) found with erythrocyte cholinesterase the following series: phenyl acetate > *p*-methoxyphenyl acetate > *p*-nitrophenyl acetate. A similar relationship was observed by us with eel esterase (Table 3). In contrast with these findings, the following order results in imidazole-catalysed hydrolysis: *p*-nitrophenyl ≫ phenyl > *p*-methoxyphenyl.

With imidazole catalysis, the rate is evidently inversely proportional to the electron density at the ethereal oxygen, and thus the observed order finds a simple explanation. In cholinesterases, the electron distribution around this oxygen atom determines the affinity for hydrogen-bonding, which increases with increasing electron density. Therefore the same structural factor has a twofold influence on the enzymic hydrolysis, namely, increasing electron density at the ethereal oxygen atom (e.g. in the *p*-methoxy derivative) produces greater ability to form a hydrogen bond, but at the same time also increases the activation energy required to break the C–O linkage. In the *p*-nitro derivative both effects are reversed. These considerations make it at least qualitatively understandable why phenyl acetate itself is superior to both its *p*-nitro and *p*-methoxy derivatives.

The results reported here may shed light on the problem of enzymic ester hydrolysis in general. In contrast with acid- or base-catalysed hydrolysis, the enzymic reaction is inhibited by both H^+ and OH^- ions. The most likely interpretation of the enzymic splitting of *O*-alkyl esters involves a dual mechanism: nucleophilic attack of imidazole nitrogen at the carbonyl carbon atom and simultaneous electrophilic attack of an 'active' hydrogen atom on the ethereal oxygen atom. The peculiar combination of these two factors explains the pH optimum, characteristic for almost all animal esterases. The enzymic mechanism is equivalent partly to acid hydrolysis, since hydrogen-bonding acts similarly to the attachment of H^+ ion. It contains also certain features of general base catalysis, since addition of imidazole to the carbonyl carbon places a negative charge on the carbonyl oxygen atom, similar to the effect of OH^- ions or related agents.

SUMMARY

1. The dependence upon pH of the hydrolysis of acetylthiocholine, phenyl acetate, *p*-nitrophenyl acetate and *p*-methoxyphenyl acetate by true cholinesterase from the electric eel has been measured.

2. The pH–activity curves of these substrates above pH 7 become largely independent of pH, whatever the length of the incubation period.

3. These results confirm the existence of the second component in the esteratic site, postulated earlier, namely the electrophilic group G_2, and indicate that the latter functions via hydrogen-bonding with the oxygen atom attached to the alkyl radical of the ester group.

4. The implications of these findings for the mechanism of enzymic hydrolysis are discussed.

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Studies in Calcification*

THE REACTION OF SOME HARD- AND SOFT-TISSUE COLLAGENS WITH 1-FLUORO-2:4-DINITROBENZENE

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It has long been considered that those factors which determine whether mesodermal connective tissue will calcify or not are present in the organic fraction of the tissue, and much attention has been directed to the function of mucopolysaccharides as a local factor in mineralization (Sobel, 1955). However, little work has been done on the properties of the protein fraction of the organic matrix and its relation to calicification. This paper describes some of the chemical and structural properties of collagen in both hard and soft mammalian connective tissues, found by the use of Sanger's (1945) fluorodinitrobenzene method, and shows in which respects the reaction of the hard-tissue collagens with fluorodinitrobenzene differ from those of the soft-tissue collagens. These differences may well be significant in understanding why soft-tissue collagen does not calcify, whereas hard-tissue collagen does so despite the almost identical amino acid composition (Bowes, Elliott & Moss, 1955; Eastoe, 1955), appearance under the electron microscope and X-ray-diffraction pictures of both proteins (Bear, 1952).

Samples of collagen obtained from ox achilles tendon, ox hide, ox bone, ox dentine, rat-tail tendon and human dentine have been studied. The collagen was prepared with a minimum of chemical treatment, as it was thought that the small amounts of reticular and elastic fibres that might be present would lead to less error than the drastic treatments which are necessary for their complete removal. In most of the experiments demineralization of bone and dentine for structural studies with fluorodinitrobenzene was effected by shaking with ethylenediaminetetra-acetic acid at pH 8·0, but in some experiments demineralization was also carried out with hydrochloric acid or trichloroacetic acid, although it was recognized that treatment with acid may damage the protein. In some experiments the tissue was treated with ethylenediaminetetra-acetic acid and fluorodinitrobenzene simultaneously, thus avoiding any time lag between demineralization and subsequent dinitrophenylation.

Preliminary accounts of some of this work have already been published (Solomons & Irving, 1955, 1956*a*, *b*).

* Some of the results quoted in this paper were taken from a thesis presented by C. C. S. for the degree of Ph.D. of the University of the Witwatersrand.

MATERIALS

Ox-hide collagen. Hide from the thigh region of a freshly killed ox was washed first with water to remove blood, etc., then left to stand overnight in 0·9% NaCl soln. at 4° and then re-washed with water. The grain layer, which contains the greater part of the muscle and elastic tissue as well as hair roots, was split off and the remaining material, excluding adipose tissue, was cut into approx. 0·5 cm. cubes. The cubes were defatted with three changes of light petroleum (b.p. 40–60°).

Ox achilles tendon and rat-tail tendon. These were cut into thin strips, washed in 0·9% NaCl soln. and defatted with light petroleum.

Ox bone. The diaphysis of an ox tibia, soaked in 0·9% NaCl soln. overnight and then washed with water, was cut into sections about 1 in. thick with a hacksaw. These sections were then split into approx. 0·25 cm. cubes by means of root-splitting dental forceps. Care was taken here and in the preparation of the dentine to minimize the generation of heat, as this can lead to denaturation as well as solution of some of the protein fraction.

Human dentine. Approx. 0·25 cm. cubes of human root dentine from healthy incisor and canine teeth were

obtained with root-splitting forceps. The pulp material was removed and the dentine soaked overnight in 0·9% NaCl soln.

Ox dentine. This was similarly prepared from ox incisor teeth.

METHODS

Demineralization of tissues. This was carried out in ethylenediaminetetra-acetic acid (EDTA) soln. (0·5 M) adjusted to pH 8·0, N-HCl or N-trichloroacetic acid. Samples (0·2 g.) of tissue were placed in 15 ml. of the decalcifying agent maintained at 4°. At regular intervals pieces of tissue were removed, allowed to dry in air, and re-weighed in order to calculate the percentage demineralization. The moisture content was determined on a separate sample by drying at 105° for 16 hr. This method was shown to be valid by estimations of calcium and phosphorus on the supernatant fluid. In order to ensure true comparisons, the soft tissues were similarly treated with demineralizing agents before dinitrophenylation.

Dinitrophenylation of tissues. The technique was as follows (Sanger, 1945): samples (0·2 g.) of tissue were treated with a mixture of 0·1 g. of $NaHCO_3$ and 0·5 g. of fluorodinitrobenzene (FDNB) in 15 ml. of 66% (v/v) ethanol for 36 hr. at room temperature. Prolonging the period of dinitrophenylation to 3 weeks had no effect on the amounts of dinitrophenyl (DNP) amino acids recovered. The DNP proteins were then washed with water and shaken with changes of acetone until no more yellow-coloured substances could be extracted. The DNP amino acids were liberated by hydrolysing the DNP protein in a sealed tube at 105° with 20 ml. of constant-boiling HCl which had been redistilled over $SnCl_2$. A hydrolysis time of 16 hr. was used since it was found that this time resulted in complete hydrolysis of the protein with minimal destruction of ε-DNP amino acids.

By repeated extraction of the acid hydrolysate with 5 ml. portions of peroxide-free ether, the ether-soluble α-DNP amino acids were separated from the water-soluble ε-DNP amino acids which remained in the acid aqueous phase. The α-DNP amino acids were separated on paper chromatograms by developing with *tert.*-amyl alcohol saturated with 0·2 M-potassium hydrogen phthalate buffer, pH 6·0 (Blackburn & Lowther, 1951). The spots thus obtained were eluted with 1% $NaHCO_3$ soln. and the optical density of the solutions was measured at 360 mμ with the Beckman spectrophotometer model B. It was found that the absorption maximum of ε-DNP lysine and hydroxylysine in N-HCl occurs at 360 mμ. The α-DNP amino acids (with the exception of α-DNP arginine and the DNP prolines) absorb maximally at 355 mμ in 1% $NaHCO_3$ soln. In this work both α- and ε-DNP amino acids were colorimetrically determined at 360 mμ and their concentrations obtained from a standard calibration curve. Interference from *O*-DNP tyrosine was negligible at this wavelength. The total concentrations of ε-DNP lysine and hydroxylysine in the acidic watery layer were similarly determined by recording the total optical density at 360 mμ. A portion of this solution was placed on a paper chromatogram and developed with butanol–acetic acid–water (4:1:5). The spots corresponding to ε-DNP lysine and ε-DNP hydroxylysine were discrete and their R_F values were 0·59 and 0·48 respectively. The spots were eluted separately with N-HCl and their optical densities measured at 360 mμ. α-DNP arginine, which would remain in the acid aqueous layer was not detected.

Recovery of dinitrophenyl amino acids. Correction factors for the destruction of DNP amino acids during hydrolysis and subsequent chromatographic procedures were obtained by treating known quantities of pure DNP amino acids for various lengths of time with 6 N-HCl at 105° in the presence of protein, and measuring the amounts remaining after chromatographic separation. After hydrolysis for 2, 8, 16 and 24 hr. the recoveries of both ε-DNP lysine and ε-DNP hydroxylysine were 98, 90, 80 and 75% respectively. The recoveries of α-DNP aspartic and glutamic acids after hydrolysis for 16 hr. were both 64%. Free lysine and hydroxylysine in hydrolysates of protein or DNP protein were determined by making use of the fact that copper ions will protect the α- but not the ε-amino groups of dibasic amino acids from reacting with FDNB (Porter, 1950). A measured volume of the hydrolysate was neutralized with excess of solid $NaHCO_3$. The solution was brought to the boil and $CuCO_3$ powder was added until no more dissolved. The solution was boiled for 2 min., filtered and the filtrate treated with FDNB (0·1 g.) for 24 hr. The mixture was then acidified and extracted with ether. The optical density of the aqueous layer, containing ε-DNP lysine and ε-DNP hydroxylysine, was measured at 360 mμ and the concentrations were obtained from a calibration curve. Care was taken to keep acidic solutions of the α-DNP amino acids in the dark, as they decompose in ultraviolet light. The ε-DNP amino acids were found to be relatively stable to light.

Combined demineralization and dinitrophenylation of hard tissues. Demineralization of hard tissues by means of EDTA and simultaneous dinitrophenylation of the protein fraction with FDNB were both carried out in one operation.

Samples (0·2 g.) of hard tissue were treated with 15 ml. of 0·5 M-EDTA (pH 8·0) containing 0·1 g. of $NaHCO_3$, to which was added 0·5 g. of FDNB dissolved in 5 ml. of ethanol. This solution was renewed daily for 5 days, after which demineralization was complete. The subsequent procedures are described above.

RESULTS

Demineralizing agents, under the conditions used, did not cause the liberation of free amino acids from any of the tissues studied, as judged by treating the supernatant solution with ninhydrin or FDNB.

Human dentine. The first experiments were carried out on human dentine, dinitrophenylation following the various stages of decalcification. In fully calcified dentine, no free *N*-terminal groups were detected. However, during demineralizing small amounts of aspartic and glutamic acids were detected and these increased in a linear manner to a maximum of 10 μmoles/g. of protein, when related to the percentage demineralization (Table 1). Hydrolytic decomposition products of DNP proline were not detected (Scanes & Tozer, 1956) and no DNP glycine was found after hydrolysis for 4 hr.

Fully calcified dentine possessed small amounts of ε-amino groups, about 25–35 μmoles/g. of protein,

i.e. about 9% of the total concentration. As demineralization progressed, the number of ε-amino groups available to FDNB increased in a linear manner to 278 μmoles/g. of protein, when plotted against the percentage decalcification (Table 1 and Fig. 1). Thus in the fully demineralized state, over 96% of the ε-amino groups reacted with FDNB, accepting the figure for total lysine and hydroxylysine obtained by Hess & Lee, 1954. Similar results were obtained with all three of the demineralizing agents employed.

Ox bone. Fully calcified ox bone had extremely small amounts of terminal aspartic acid, about 1–2 μmoles/g. of protein, and these remained constant during demineralization. Fully calcified ox bone had 35 μmoles of ε-amino groups/g. of protein. When decalcification was followed by dinitrophenylation, the number of ε-amino groups available to FDNB at complete decalcification varied very considerably from sample to sample, ranging from 55 to 100% of the total number present. It seemed possible that the procedure of simultaneous decalcification and dinitrophenylation would yield more consistent results, as the reaction of the ε-amino groups would follow immediately on decalcification. When this was done, the availability of ε-amino groups to FDNB increased in a linear manner to 330 μmoles/g. of protein, when plotted against the percentage demineralization (Fig. 1). Thus in the fully demineralized state, over 93% of the ε-amino groups reacted with FDNB, by accepting the value for the total lysine and hydroxylysine content of bone obtained by Eastoe (1955).

Table 1. *α- and ε-amino groups of human dentine collagen*

Liberated groups were estimated by reaction with FDNB during decalcification with EDTA at room temperature (pH 8·0).

Percentage demineralization	Groups (μmoles/g. of protein)
α-Amino groups	
0	0·0
20·0	2·1
38·8	4·0
55·0	5·8
75·0	7·7
100·0	10·2
ε-Amino groups*	
0	28
18·8	60
32·6	100
45·0	135
57·0	165
75·0	210
90·1	245
100·0	278

* Total content = 289 μmoles/g. (Hess & Lee, 1954).

In view of these findings, simultaneous decalcification and dinitrophenylation were carried out later on human dentine, but the results with this procedure were the same as those obtained when the processes were successive.

Ox dentine. The results obtained were the same if decalcification and dinitrophenylation were simultaneous or successive. No free *N*-terminal amino acids were detected in the calcified tissue, but after demineralization 2–4 μmoles of terminal aspartic acid/g. of protein were found. Fully calcified ox dentine had about 25–30 μmoles of ε-amino groups/g. of protein, and as with human dentine these rose linearly during demineralization to a maximum of 340 μmoles/g. of protein when decalcification was complete (Fig. 1).

Soft tissues. No *N*-terminal amino acids were detected in ox-hide collagen, ox achilles tendon or rat-tail tendon in the native state, and no *N*-terminal amino groups were liberated when these tissues were treated with demineralizing solutions under the same conditions as were used to decalcify the hard tissues.

Between 64 and 70% of the total number of lysyl and hydroxylysyl ε-amino groups present in these soft tissues were recovered as their DNP

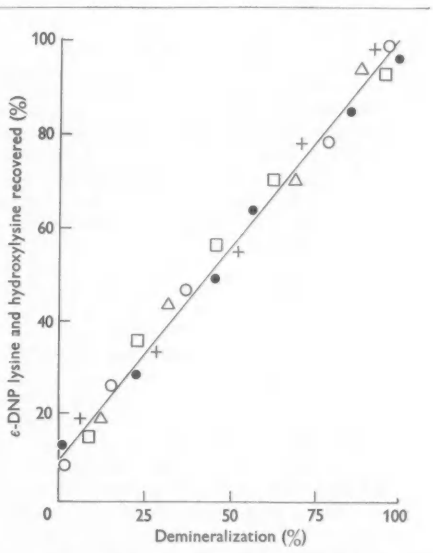

Fig. 1. Availability of ε-lysyl and hydroxylysyl amino groups of hard-tissue collagens to FDNB during demineralization. ○, ●, Denote successive demineralization and dinitrophenylation of ox dentine and human dentine respectively; +, □, △, denote simultaneous demineralization and dinitrophenylation of ox dentine, ox bone and human dentine respectively.

Table 2. *Free ε-amino groups of soft-tissue collagens*

Tissue	Total content (μmoles/g. of protein)	Percentage of total content reacting with FDNB		
		Mean	S.E.	No. of determinations
Ox hide	341*	65·0	±1·2	20
Ox achilles tendon	341†	67·0	±1·5	10
Rat-tail tendon	340†	68·0	±1·3	12

* Bowes, Elliott & Moss (1955).
† Results obtained by the present writers with the copper method (see text).

derivatives after treatment with FDNB (Table 2). The percentage of ε-amino groups available to FDNB was not changed either by increasing the pH to 10 during coupling with FDNB or by performing the reaction in aqueous medium. As has been reported by other investigators, modification of ox-hide collagen by hydrothermal shrinkage, or treatment with urea or acetic acid or alkali, did not change the availability of ε-amino groups.

The remaining lysyl and hydroxylysyl residues reacted quantitatively with FDNB only after hydrolysis of the DNP protein. In this way it was possible to account for over 95% of the lysine and hydroxylysine originally present in each protein. Treatment with demineralizing agents did not affect these results.

DISCUSSION

The most significant findings from these investigations are that the collagens of soft and demineralized hard tissues can be readily distinguished from each other by the reactivity of their ε-lysyl and hydroxylysyl amino groups with FDNB. That a linear relationship exists between the release of these groups and the degree of demineralization (with several different decalcifying agents) strongly suggests that there is a fundamental connexion between free ε-amino groups of collagen and calcification. The lower reactivity of the soft-tissue collagens is probably not due to steric factors, as swelling the protein with urea solutions had no effect on the yield of DNP amino acids. Bowes & Moss (1953) treated ox-hide collagen with FDNB and observed the low yield of DNP lysine. They postulated that this might be due to ε-DNP lysine being less stable to acid when combined in the collagen than when present as the free amino acid. On hydrolysis of the DNP protein they recovered only small amounts of free lysine; this result is at variance with those of the present authors, who were able to account for almost all the lysine and hydroxylysine originally present in the protein. It appears that ε-lysyl groups may take part in peptide cross-links, since Mechanic (1957) has recently isolated an ε-lysyl tripeptide from a partial acid hydrolysate of ox achilles tendon. This possibly explains the failure of some of the ε-amino groups to react with FDNB.

The absence of *N*-terminal amino groups in all the soft-tissue collagens studied is in agreement with the results obtained by others on ox-hide collagen (Bowes & Moss, 1953). This finding indicates that the molecule either is extremely large or is cyclic or that the terminal amino groups may be masked in some way. The amounts of *N*-terminal amino groups present in ox bone and ox dentine are so small that it is difficult to attach any significance to them. Demineralized human dentine contains amounts which, though not large, are appreciable. Only in human dentine was there any correlation between the concentration of free terminal amino groups and the degree of demineralization. Too much significance should not, however, be attached to the absolute values for the concentrations of *N*-terminal residues in human dentine until more is known about the nature of the polypeptide chains that form dentine collagen. It is possible that human dentine may have a chain length smaller than that of bovine dentine or bone.

It is not unlikely that dentine and bone collagens differ in some details of their intimate structure since consistent values for the full availability of the ε-amino groups to FDNB could only be obtained with bone if demineralization and dinitrophenylation were carried out simultaneously.

In previous studies of calcification, attention has been directed more to the role that mucopolysaccharides play in this process (Sobel, 1955). Such work has been done almost entirely with rachitic hypertrophic cartilage in which the content of chondroitin sulphate is high. Bélanger (1954) has found that ^{35}S is incorporated into bone and dentine during calcification, and has interpreted his results to indicate that chondroitin sulphate is also concerned with calcification in these tissues, though their content of mucopolysaccharide is very low. Whereas it is possible that such substances as chondroitin sulphate are concerned with calcification, the present work indicates that the nature of collagen is also an important factor. Since calcification is a process of crystallization rather than precipitation (Neuman & Neuman, 1953), hard-tissue collagens, in which all the ε-lysyl and

hydroxylysyl amino groups are free to react with FDNB, may act as a suitable 'seeding template' for the growth of hydroxyapatite crystals. The fact that apatite crystals are deposited on collagen fibres predominantly at the cross-banded regions (Robinson & Watson, 1955), where it is thought that the content of basic and acidic amino acids is high, supports this concept.

SUMMARY

1. A variety of hard and soft connective tissues of bovine and human origin were treated with fluorodinitrobenzene before, during and after treatment with demineralizing agents.

2. It was found that the collagens of soft and demineralized hard tissues could readily be distinguished from each other on the basis of the reactivity of their ϵ-lysyl and hydroxylysyl amino groups with fluorodinitrobenzene. In intact soft-tissue collagen only about two-thirds of these ϵ-amino groups reacted with fluorodinitrobenzene, but the concentration of reactive ϵ-amino groups of hard-tissue collagens increased from very low levels almost to the theoretical value at complete decalcification.

3. The significance of these findings is discussed in relation to calcification *in vivo*.

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Studies in Carotenogenesis

24*. THE CHANGES IN CAROTENOID AND CHLOROPHYLL PIGMENTS IN THE LEAVES OF DECIDUOUS TREES DURING AUTUMN NECROSIS

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It has long been stated that the change in colour of the leaves of deciduous trees in early autumn is due to the preferential destruction of the green chlorophylls, which unmasks the much more slowly disappearing yellow carotenoids. Early work indicated that the carotenoids also undergo qualitative as well as quantitative changes. From 70 to 80% of the total carotenoids in all green leaves are unesterified xanthophylls (Goodwin, 1952), which are hypophasic when partitioned between light petroleum and 90% (v/v) aqueous methanol (Goodwin, 1955). Tswett (1911) found that the carotenoids from autumn leaves were almost entirely epiphasic, but that the major fraction could be separated from β-carotene; he named these pigments 'autumn xanthophylls'; Palmer (1922), considering their partition properties, suggested the name 'autumn carotenes'. Kuhn & Brockmann (1932) found that these autumn pigments were not hydrocarbons but esterified xanthophylls, which behaved like carotenes in the partition test. Karrer & Walker (1934), however, considered that they were unidentified oxidation and degradation products of lutein. On the other hand, Strain (1938) stated that the major pigment in yellow-shed leaves was zeaxanthin (3:3′-dihydroxy-β-carotene). Kuhn & Brockmann (1932) agreed with Willstätter & Stoll (1918) that the total amount of carotenoids did not alter in autumn leaves; however, Karrer & Walker (1934) and Nagel (1939) found that both the carotenes and xanthophylls disappeared, but that the carotenes were destroyed more quickly than xanthophylls. The carotenoids are reported to undergo these same autumnal changes in leaves of tropical evergreens (Egle, 1944). The *aurea* varieties of *Acer*

* Part 23: Goodwin, Land & Sissins (1956).

laetum and *Buxus japonica* during the period from late May to late October tended to increase their carotene levels whilst maintaining their xanthophyll levels constant; in *Ulmus montana* var. *aurea*, however, both levels remained constant during the same period, except that in October a sharp decline, much more marked in the carotene fraction, had set in (Bauer, 1956).

Because of the various inconsistencies in the literature and because most of the work had been done in the early days of investigations on carotenoids, it was decided to use the newer techniques and knowledge now available to follow the changes in carotenoid content of the leaves of three deciduous trees. Those chosen were the common sycamore (*Acer pseudoplatanus*), the oak (*Quercus robur*) and the cultivated black plum (*Prunus nigra*). The first two were chosen because sycamore leaves turn yellow much earlier than oak leaves, and the last because it contains, in addition to the chloroplast pigments, a cytoplasmic anthocyanin. In addition qualitative and quantitative changes in the chlorophylls and anthocyanin levels present were also investigated. Willstätter & Stoll (1918), in their classical experiments, considered that chlorophylls *a* and *b* disappeared at the same rate; on the basis of recent work with more refined techniques there are indications that chlorophyll *a* is destroyed somewhat faster than chlorophyll *b* (Rudolph, 1934; Nagel, 1939; Egle, 1944; Seybold, 1943; Wolf, 1956). There appears to be little known about the changes in anthocyanin levels during the autumn.

EXPERIMENTAL

Materials. The same tree of each of the three genera examined was used throughout. *Acer pseudoplatanus* (sycamore) and *Quercus robur* (oak) were growing in a small copse in north-west Cheshire and *Prunus nigra* (black plum) in a nearby garden. Their leaves were collected during 1956, always from the same region of the tree, between 8 and 9 a.m., and transported to the Laboratory in a polythene container. Extraction of the pigments began within 30–45 min. of collecting the leaves.

Extraction of chlorophylls and carotenoids. The leaves (between 0·5 and 2·0 g.) were ground in a mortar under acetone (25 ml.) with acid-washed silver sand, and the mass was filtered through a sintered-glass filter (G 1). The residue was returned to the mortar and re-extracted with acetone; this process was repeated (usually three times) until the final acetone extract was colourless. The acetone extracts were combined in a separating funnel and transferred to diethyl ether by adding approximately an equal volume of that solvent, followed by water added dropwise until two layers formed. The lower layer was run off, the ether layer was washed free from acetone with water (3 × 20 ml.), and dried by standing for 30 min. over anhydrous Na_2SO_4, filtered and made up to a known volume (usually 100 ml.). This was then divided into four portions: (*A*) 50 ml. was taken to dryness under N_2 and dissolved in a small volume of light petroleum (b.p. 40–60°) for column chromatography; (*B*) 25 ml. was taken for determination of total carotenoids; (*C*) 20 ml. was taken for paper chromatography; (*D*) 4–5 ml. was required for the direct spectrophotometric determination of chlorophylls.

Extraction of anthocyanin. With *P. nigra* a small amount of anthocyanin pigment was removed in the first acetone extraction; this was ignored. The major portion, representing at least 95% of the total, was present in the material remaining after treatment with acetone. The most suitable solvent for extraction was found to be ethanol containing 1% (v/v) HCl. The extraction was carried out in the same way as for the carotenoids. The residue, already containing silver sand, was transferred from the sintered-glass filter to the mortar and ground under the acid ethanol (25 ml.). The mass was filtered through the sintered-glass filter and the residue returned to the mortar for re-extraction; three extractions were usually sufficient for complete removal of the pigment. The extracts were combined and made up to a known volume for spectrophotometric analysis.

Separation of the chloroplast pigments by column chromatography. The adsorbent found most suitable for the separation of the major chloroplast pigments was (confectioner's) icing sugar (Strain, 1940; Zechmeister & Cholnoky, 1943; Lederer & Lederer, 1953; Goodwin, 1955). The icing sugar was dried in a vacuum desiccator overnight before use. A column (2 cm. × 12·5 cm.) was prepared in the dry state (Zechmeister & Cholnoky, 1943) and light petroleum (the fraction boiling between 40° and 60° was used throughout this investigation) drawn through under slight suction. The pigment extract from 0·125–0·5 g. of fresh leaves was placed on the column in 5–10 ml. of light petroleum and the chromatogram developed with the same solvent until all the β-carotene, which is not adsorbed on icing sugar, had run through the column. This fraction was then made up to a volume suitable for spectrophotometric measurements. The remaining pigments were then separated in one of two ways: (*a*) by a 'liquid chromatogram' in which the developing solvent (light petroleum) was made increasingly polar by addition of acetone and the fractions were collected consecutively in the eluate as they moved off the column; (*b*) by developing the column with light petroleum containing 0·5–1·0% (v/v) of acetone until discrete zones were formed on the column; about 200–250 ml. of eluent was usually required. The residual solvent was removed from the column by suction, the column extruded and the zones were separated by cutting the column at the appropriate points. The pigment from each zone was then eluted by shaking with diethyl ether (25 ml.) and filtering through a sintered-glass funnel. The fractions were taken to dryness under N_2 and dissolved in appropriate volumes of light petroleum for spectrophotometric examination.

Separation of chloroplast pigments by paper chromatography. The pigments were separated by ascending paper chromatography with Whatman no. 1 paper and the solvent system of Loeffler (1955), which is light petroleum–ethyl ether–ethanol (30:10:0·5, by vol.). It was found necessary to have the chromatography vessel lined with Whatman no. 1 paper dipping in the solvent and for the solvent to reach the top of the paper before inserting the chromatogram. In this way the space was kept saturated with

solvent vapour. The portion of the pigment extract retained for paper chromatography (20 ml.) was taken to dryness and dissolved in ethanol (1 ml.); 0·05 ml. of this was applied to the paper in small portions, each addition being allowed to dry at room temperature before application of the next portion. Drying by heating destroys the pigments. After separation, the paper was dried in air and the spots were cut out, eluted with ethyl ether and examined spectrophotometrically in that solvent. Chromatograms were always run in duplicate.

Quantitative determination of carotenoids. Total carotenoids were determined by evaporating a known volume of the crude ether extract of the leaves to dryness and removing the chlorophylls by saponifying the extract according to the method of Goodwin & Morton (1946). This destroys the chlorophylls and leaves the carotenoids in the unsaponifiable fraction. The unsaponifiable matter is dissolved in a known volume of light petroleum and the total carotenoid content obtained from measuring E (446 mμ), assuming $E^{1\%}_{1\,cm.}$ for the crude mixture to be that of β-carotene (2500; Goodwin, 1955).

The concentration of the various components obtained after chromatography was obtained similarly by measuring $E_{max.}$ and using the appropriate $E^{1\%}_{1\,cm.}$ value: β-carotene, 2500 at 449 mμ; lutein, 2500 at 445 mμ; neoxanthin, 2270 at 435 mμ; violaxanthin, 2550 at 441 mμ; lutein-5:6-epoxide, 2400 at 441 mμ (Goodwin, 1955).

Quantitative determination of chlorophylls. The concentrations of chlorophyll *a* and chlorophyll *b* were determined in the crude ether extract of the leaves by differential spectrophotometry, with the equations developed by Smith & Benitez (1955): $C_a = (0{\cdot}0101E_{662}) - (0{\cdot}00101E_{644})$ and $C_b = (0{\cdot}0164E_{644}) - (0{\cdot}00257E_{662})$, where C_a and C_b are the concentrations of chlorophyll *a* and chlorophyll *b* in g./l. and E_{644} and E_{662} are the extinction measurements in a 1 cm. cell, at 644 and 662 mμ respectively.

Direct spectroscopic measurements were also made on the chlorophyll *a* and chlorophyll *b* fractions obtained by column and paper chromatography; the spectroscopic characteristics reported for these pigments by Smith & Benitez (1955) were used. The ratio of chlorophyll *a*:*b* obtained by direct spectrophotometry was always similar to that found by using the chromatographic methods, except in the later samples when the small amounts of chlorophylls remaining in the leaves had obviously been altered so that their absorption spectra had changed and the equations were no longer applicable.

Determination of the anthocyanin in P. nigra *leaves.* The anthocyanin ran as a single spot on paper chromatography with butan-1-ol–acetic acid–water (4:1:5, by vol.) and it is almost certainly cyanidin-3′-glucoside (see Results). Its concentration was determined by measuring E at 538 mμ, assuming the $E^{1\%}_{1\,cm.}$ of cyanidin-3-glucoside to be 700 (Geissman, 1955).

Dry-weight determinations. These were carried out by drying the leaves overnight at 80° in an air oven.

Isolation of chloroplasts. The leaves (2 g.) were thoroughly ground up at 5° with acid-washed silver sand under 0·5 M-sucrose (50 ml.) (Granick, 1938). The cell debris was centrifuged off at 500 *g* and the chloroplasts were collected by centrifuging at 1000 *g*. Fractions were collected half-hourly for 1·5 hr.

Spectrophotometric measurements. These were carried out with a Unicam spectrophotometer model SP. 600.

RESULTS

Nature of the pigments

Chlorophylls. It has been accepted for many years that only chlorophyll *a* and chlorophyll *b* are found in the green tissues of higher plants (Smith & Benitez, 1955); these were the only pigments found in the green leaves now under examination. In yellow autumn leaves the small amounts remaining had chromatographic properties very similar to those of authentic chlorophylls *a* and *b*, but differed from these slightly in their absorption maxima. These 'changed chlorophylls' had their absorption maxima (light petroleum) in the red at 652 mμ for chlorophyll *a* and at 635 mμ for chlorophyll *b* instead of at 662 mμ and 644 mμ respectively.

In yellow leaves, traces of pale-grey zones occasionally appeared just below the 'changed' chlorophylls *a* and *b*. These are phaeophytins *a* and *b* (Smith & Benitez, 1955). Because of their sporadic occurrence, it would be premature to conclude at this stage that they were intermediates in the degradation of chlorophylls *in vivo*; they can easily arise from the parent compounds during manipulation, especially in the presence of traces of acid.

Carotenoids. It has long been accepted that β-carotene is the major carotene of green leaves and that it is accompanied by varying but smaller amounts of α-carotene and traces of *cis*-isomers of α- and β-carotene (Goodwin, 1952). It has only recently been shown that the xanthophyll mixture is also comparatively simple, the earlier reports of complex mixtures being due to changes brought about during chromatography by traces of free HCl liberated from ethylene dichloride used as eluent (Strain, 1938, 1948). Some 90–95 % of the total xanthophylls of lucerne (alfalfa) (Bickoff, Livingston, Bailey & Thompson, 1954) and maize (corn) (Moster, Quackenbush & Porter, 1952) are represented by lutein (3:3′-dihydroxy-α-carotene), violaxanthin (5:6:5′:6′-di-epoxyzeaxanthin) and neoxanthin (structure unknown). Small amounts of cryptoxanthin (3-hydroxy-β-carotene, zeaxanthin and flavoxanthin (5:8-epoxylutein) are also occasionally encountered. These pigments had been repeatedly noted in earlier work but additional artifacts were also described.

In the three types of green leaves examined here β-carotene, lutein, violaxanthin and neoxanthin were the major components. Table 1 illustrates a typical chromatogram obtained with icing sugar as adsorbent. Violaxanthin was not separable from chlorophyll *a* with this adsorbent and its presence was established by removing the chlorophyll *a* zone, saponifying the extract to destroy the chlorophyll *a*

and rechromatographing the yellow unsaponifiable extract on icing sugar. This yielded a single band corresponding in position and absorption spectrum to violaxanthin.

In autumn leaves, the extracts of plum and oak leaves contained β-carotene (traces), lutein, violaxanthin but no neoxanthin. A new pigment was found in small amounts adsorbed just below violaxanthin and chlorophyll *a*; as this pigment is hypophasic to 90 % aqueous methanol and has an absorption spectrum very similar to violaxanthin but a lower adsorptive affinity, it is probably lutein 5:6-epoxide (Goodwin, 1955).

In addition to lutein, violaxanthin and lutein epoxide, already noted in plum and oak leaves, two further pigments were observed in autumn leaves of the sycamore. These were adsorbed between β-carotene and lutein. They were epiphasic in the phase tests, indicating no free hydroxyl groups, but on saponification they became hypophasic and were identified by their absorption spectra and by mixed chromatography with authentic specimens as violaxanthin and lutein respectively. These new pigments were obviously the 'autumn xanthophylls' of Tswett (1911), and were correctly identified as xanthophyll esters by Kuhn & Brockmann (1932).

Paper chromatography with Loeffler's (1955) solvent system is effective for separating the major pigments in leaves, and Fig. 1 shows the different chromatograms obtained with the extracts of green and yellow sycamore leaves.

Anthocyanin. The anthocyanin present in *P. nigra* has not previously been described but all other species examined (*P. persica*, *P. divaricata* var. *pissardii*, *P. lannesiana* var. *donarium*, *P. nipponica*, *P. avium*, *P. pissardii*) contain a cyanidin-3-monoside, most probably cyanidin-3-glucoside (chrysanthemin) (Lawrence, Price, Robinson & Robinson, 1939; Hayashi & Abe, 1953; Reznik, 1956). The pigment in *P. nigra* is also almost certainly a cyanidin monoside, it has the same absorption spectrum as the pigment from *P. pissardii* and cannot be separated from it on paper chromatography with butanol–acetic acid–water.

Table 1. *A typical chromatogram of the major pigments of green leaves*

Adsorbent: icing sugar; column size: 2 cm. × 12·5 cm.; pigments from 0·125 to 0·5 g. of fresh leaves; developer: light petroleum containing 0·5–1·0 % of acetone. Zones are in order of increasing adsorptive affinity.

Zone no.	Description	Pigment
1	Orange, passing straight through the column	β-Carotene*
2	Yellow	Lutein
3	Light green	Chlorophyll *a*† violaxanthin
4	Dark green	Chlorophyll *b*
5	Lemon yellow	Neoxanthin

* Possible traces of α-carotene are not separated from β-carotene by this adsorbent.

† These two pigments cannot be fully separated on this adsorbent.

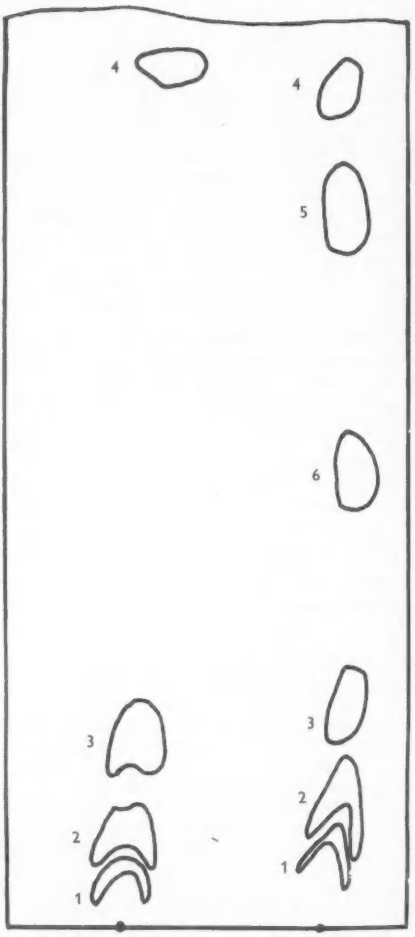

Fig. 1. Paper chromatograms of pigments extracted from green and yellow sycamore leaves. 1, Chlorophyll *b*; 2, chlorophyll *a* + violaxanthin; 3, lutein; 4, β-carotene; 5, lutein esters; 6, violaxanthin esters. Ascending chromatogram was run at room temperature on Whatman no. 1 paper with light petroleum–ethyl ether–ethanol (30:10:0·5, by vol.) as solvent system (Loeffler, 1955). Only traces of β-carotene were present in the extract from yellow leaves.

Quantitative observations

Oak leaves. The variations in percentage dry weight, total chlorophyll and total carotenoid are given in Fig. 2. From this it is obvious that both the chlorophylls and carotenoid decreased precipitately from the middle of September to the middle of October. There is no indication in these leaves that the chlorophylls are destroyed before the carotenoids. The values recorded here for the total carotenoids of the green leaves (11–15 mg./100 g. dry wt.) are within the range previously recorded for *Quercus* spp. by Kemmerer, Fudge & Fraps (1942). The relative changes in the carotenoid components are recorded in Fig. 3. During the period when the sharp drop in the total carotenoid level is occurring (see Fig. 2), the major changes are (i) the rapid and complete disappearance of neoxanthin, (ii) the appearance of lutein-5:6-epoxide for the first time, (iii) the relatively more rapid disappearance of β-carotene compared with that of lutein, which results in the latter being the major component in late leaves, and (iv) the comparative constancy of the violaxanthin fraction. β-Carotene-5:6-epoxide could not be detected in significant amounts; if it is an intermediate in the breakdown of β-carotene, it is presumably rapidly destroyed as soon as formed. The relative amounts of the constituent carotenoids in the green oak leaves fall within the normal values for green leaves (Goodwin, 1952).

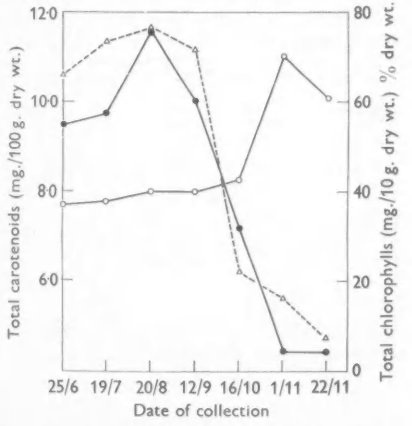

Fig. 2. Variations in dry weight, total chlorophyll and total carotenoid values for oak leaves during summer and autumn. ●, Total chlorophylls; △, total carotenoids; ○, dry weight. Samples collected on 1 and 22 November were shaken off the branches. Chlorophylls in these samples were 'changed chlorophylls'. In this and other figures the dates are represented, for example, as 1/11 (meaning 1 November).

The chlorophyll *a*:*b* ratios during necrosis are given in Table 2. Until the chlorophylls have almost completely disappeared from the leaf there is only a slight tendency of chlorophyll *a* to be destroyed more quickly than chlorophyll *b*. In the very late stages, when the traces of pigments remaining are no longer true chlorophylls *a* and *b*, the chlorophyll *a* derivative disappears much more rapidly than the chlorophyll *b* derivative.

Plum leaves. The variations in percentage dry weight, total chlorophyll, total carotenoid and total anthocyanin are given in Fig. 4. Here again there is a sharp drop in chlorophyll levels during early October, but it is very much less marked than with the oak (Fig. 2) or sycamore (see later). The drop in carotenoids is much more gradual, occurring throughout the period under observation; it did

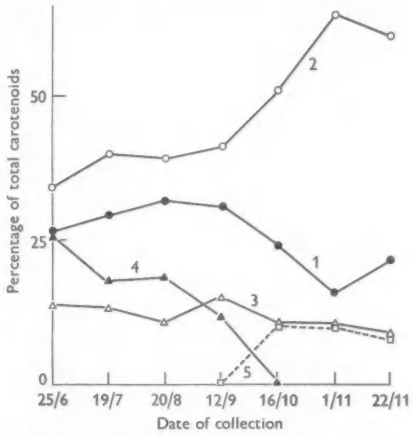

Fig. 3. Changes in the relative amounts of carotenoids in oak leaves during summer and autumn. 1, β-Carotene; 2, lutein; 3, violaxanthin; 4, neoxanthin; 5, lutein 5:6-epoxide.

Table 2. *Chlorophyll* a:*chlorophyll* b *ratio in oak leaves during summer and autumn*

Ratios were determined in an ethereal solution by differential spectrophotometry (see Experimental section) and were checked by paper and column chromatography.

Date	Ratio *a*:*b*	Date	Ratio *a*:*b*
25 June	2·60	16 October	2·35
19 July	2·72	1 November*	1·67
20 August	2·81	22 November*	1·08
12 September	2·43		

* The chlorophylls in these two specimens were 'changed chlorophylls' (see text) and therefore the ratios could not be determined spectrophotometrically; they were determined chromatographically.

not parallel the disappearance of chlorophylls but had begun before the autumnal drop in chlorophylls. The final 'autumn' levels of chlorophylls and carotenoids reached were much higher than in the oak (Fig. 2) or sycamore (see later). The increase in percentage dry weight in early autumn is also much less marked than in oak and sycamore leaves. The anthocyanin level does not show any downward trend during the period studied. The chlorophyll *a*:*b* ratio (Table 3) remained constant until the latter part of October, when it dropped significantly. The relative amounts of the constituent carotenoids, which in early summer are within the normal levels for green leaves, alter with the onset of autumn in the same way as observed with oak leaves (Fig. 5). When the carotenoids begin to disappear the β-carotene and neoxanthin are preferentially destroyed; this is especially marked with the latter. Lutein is much more stable and lutein 5:6-epoxide makes its appearance.

Sycamore leaves. The general variations in percentage dry weight, total chlorophyll and total carotenoid are given in Fig. 6. Here the fall in chlorophylls and carotenoids is as marked as in oak leaves, and more marked than in plum leaves. The chlorophylls tend to disappear before the carotenoids and these changes begin much earlier than in the other two leaves studied; they are well advanced at the beginning of August. Dry-weight percentages rise very sharply as the leaves begin to necrose. Table 4 shows that, as in the other two cases, the chlorophyll *a*:*b* ratio drops during necrosis but the fall is marked only in the later stages. When the relative changes in the constituent carotenoids are considered, sycamore leaves are similar to the other leaves examined in that β-carotene and neoxanthin are the first pigments to disappear. However, by contrast, new pigments appear which have been identified as esters of lutein and violaxanthin. Fig. 7 shows how the relative amounts of these increase during necrosis, so that at the time the leaves fall from the trees they are the only carotenoids present. When the esters first appear they consist of almost equal amounts of lutein and violaxanthin esters; as the leaves become older, the lutein esters tend to

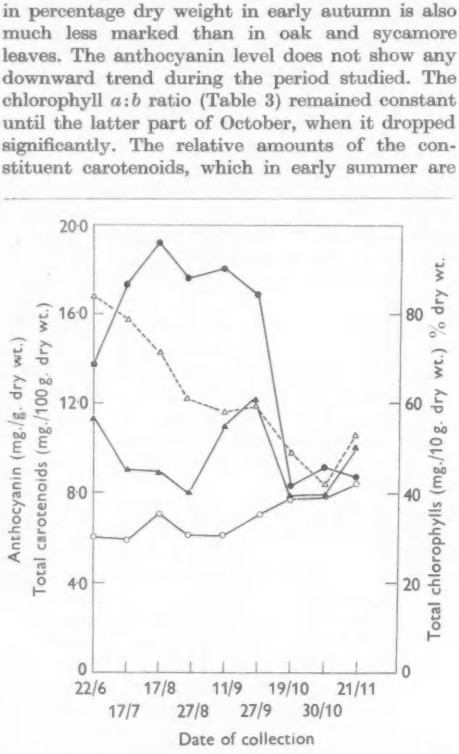

Fig. 4. Variations in dry weight, total chlorophyll, total carotenoid and anthocyanin values for plum (*Prunus nigra*) leaves during summer and autumn. ●, Total chlorophyll; △, total carotenoids; ○, dry weight; ▲ anthocyanin.

Table 3. *Chlorophyll* a:*chlorophyll* b *ratio in plum leaves during summer and autumn*

Ratios were determined in an ethereal solution by differential spectroscopy (see Experimental section) and were checked by paper and column chromatography.

Date	Ratio *a*:*b*	Date	Ratio *a*:*b*
22 June	2·39	27 September	2·18
17 July	2·58	19 October	2·33
17 August	1·90	30 October*	1·83
27 August	2·66	21 November*	1·70
11 September	2·70		

* 'Changed' chlorophylls; see footnote to Table 2.

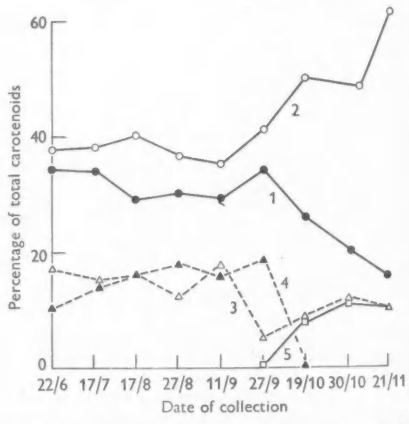

Fig. 5. Changes in the relative amounts of carotenoids in plum leaves during summer and autumn. 1, β-Carotene; 2, lutein; 3, violaxanthin; 4, neoxanthin; 5, lutein 5:6-epoxide.

disappear more quickly than violaxanthin esters, so that in fallen leaves the very small amount of residual carotenoid appears to be almost entirely violaxanthin esters (Table 5), but traces of the lutein esters may also be present.

Stability of pigments in harvested autumn leaves

Leaves from the three trees examined were collected during early October, stored in the Laboratory at room temperature and examined periodically. It was found that the pigment changes in these leaves were similar to those observed with leaves left on the tree; the results are therefore not recorded here.

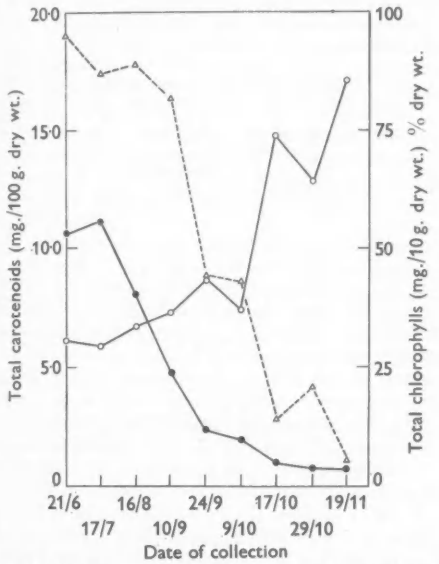

Fig. 6. Variations in dry weight, total chlorophyll and total carotenoid for sycamore leaves during summer and autumn. ●, Total chlorophyll; ○, dry weight; △, total carotenoids.

Table 4. *Chlorophyll* a:*chlorophyll* b *ratio in sycamore leaves during summer and autumn*

Ratios were determined in an ethereal solution by differential spectroscopy (see Experimental section) and were checked by paper and column chromatography.

Date	Ratio *a*:*b*	Date	Ratio *a*:*b*
21 June	3·38	9 October	1·88
17 July	2·77	17 October*	1·53
16 August	3·03	29 October*	1·51
10 September	2·44	19 November*	1·36
24 September	2·40		

* 'Changed' chlorophylls; see footnote to Table 2.

Location of xanthophyll esters in sycamore leaves

As only minute traces, if any, of esterified xanthophylls exist in the chloroplasts of the higher plants (Goodwin, 1952), it was of interest to know in which region of the cell the newly appearing xanthophyll esters occurred in necrosing sycamore leaves.

Chloroplasts were prepared according to the method of Granick (1938), and these and the supernatant were examined for carotenoids. In Table 6, it will be seen that with yellowing sycamore leaves the early fraction which would contain predominantly unbroken chloroplasts contains relatively little xanthophyll esters (less than 10%) and this might well be due to contamination with particles

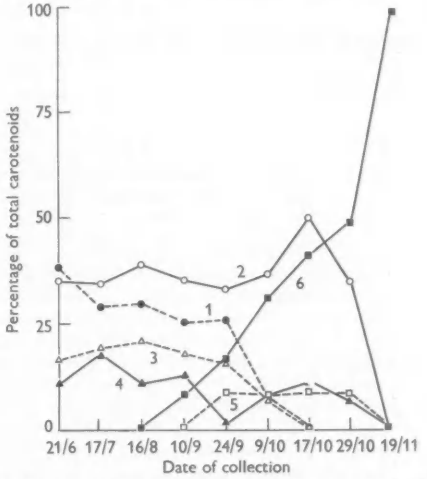

Fig. 7. Changes in dry weight, total chlorophyll and total carotenoid for sycamore leaves during summer and autumn. 1, β-Carotene; 2, lutein; 3, violaxanthin; 4, neoxanthin; 5, lutein epoxide; 6, xanthophyll esters.

Table 5. *Relative amounts of violaxanthin and lutein esters in necrosing sycamore leaves*

The period covered is that from the first appearance of xanthophyll esters to the time the leaves were falling from the trees.

Date	Percentage of total esters present: Lutein esters	Violaxanthin esters
10 September	59	41
24 September	45	55
9 October	45	55
17 October	51	49
29 October	36	64
19 November	Trace	Almost all

Table 6. *Relative distribution of free xanthophylls and xanthophyll esters in cytoplasm and chloroplasts of yellowing sycamore leaves*

Chloroplasts were obtained by the method of Granick (1938). After removal of cell debris by centrifuging at 500 *g* for 30 min., the preparation was centrifuged for 1·5 hr. at 1000 *g*, the centrifugates being collected every 15 min.

Fraction	Percentage of total pigment: Free xanthophylls	Percentage of total pigment: Xanthophyll esters
A. Centrifugate obtained during first 15 min. centrifuging at 1000 *g*	92, 91*	8, 9
B. Centrifugate obtained during final 15 min. centrifuging at 1000 *g*	42	58
C. Supernatant	36	64

* Duplicate determinations on the same preparation.

Table 7. *Relative distribution of free xanthophylls and xanthophyll esters in cytoplasm and chloroplasts of brown sycamore leaves*

Chloroplasts were obtained by the method of Granick (1938). After removal of cell debris by centrifuging at 500 *g* for 30 min., all the chloroplasts were removed by centrifuging at 1000 *g* for 30 min. No further material was obtained on further centrifuging.

Fraction	Percentage of total pigment: Free xanthophylls	Percentage of total pigment: Xanthophyll esters
A. Centrifugate obtained during first 30 min. centrifuging at 1000 *g*	51	49
B. Supernatant	Trace	100

from disintegrating chloroplasts. The last fraction obtained from centrifuging was very little different from the supernatant. This indicates that this fraction probably contained very few intact chloroplasts but consisted mainly of fragments of chloroplasts. If brown leaves are used the results are even clearer (Table 7). No lutein exists in the supernatant but it is the major pigment in the chloroplasts. If the supernatant is spun at 12 000 *g* the pigments remain unsedimented. In normal green leaves there are no detectable amounts of carotenoids in the supernatant. The most reasonable explanation of these observations is that esterification of the esters occurs only when the chloroplasts are disintegrating, that is when the pigments are exposed to the cytoplasm, which probably contains an esterase.

DISCUSSION

The present study indicates that the metabolism of carotenoids in autumn leaves cannot be considered as one process common to all deciduous trees. The previously accepted general picture was that the chlorophylls first disappeared, thus unmasking the carotenoid pigments which then gradually disappeared in the late autumn; as they disappeared the carotenoids were converted into complex oxidation products, according to Karrer & Walker (1934), or into xanthophyll esters (Kuhn & Brockman, 1932). The preferential loss of chlorophyll is well demonstrated in the sycamore (Fig. 6), but in the oak (Fig. 2) the carotenoids and chlorophylls disappear simultaneously, and in the black plum (Fig. 4) the carotenoids are preferentially destroyed. *P. nigra* differs from the other two in that the quantitative disappearance of both groups of pigments is very much less; whereas in the oak and the sycamore the falling leaves contain only traces of pigments, falling plum leaves still contain at least 50 % of their original pigments. It will be interesting to see whether future work will indicate that this is a characteristic of leaves containing anthocyanins in their cytoplasm.

The qualitative changes in the carotenoids were rather less marked than was expected and some were common to all three species. In all the onset of necrosis was characterized by the preferential destruction of β-carotene [confirming the observations of Karrer & Walker (1934) and Nagel (1939)] and neoxanthin and by the appearance of lutein-5:6-epoxide. This is probably the first stage in the oxidative degradation of lutein, because in isolated tomato leaves β-carotene epoxides are the first breakdown products of β-carotene (Glover & Redfearn, 1953). The failure to detect more than traces of β-carotene epoxides in autumn leaves is probably due to their extreme lability, which

would also account for the rapid disappearance of β-carotene. The lability of β-carotene in leaves compared with that of lutein, which appears to be a general phenomenon, is most striking; apart from the fact that it disappears rapidly from autumn leaves, it also rapidly disappears from isolated tomato leaves kept in darkness in the absence of carbon dioxide, although the xanthophyll levels remain constant; on illumination β-carotene returns to its normal level (Glover & Redfearn, 1953). Furthermore, on illuminating etiolated maize seedlings β-carotene is rapidly synthesized whereas the xanthophyll levels alter only slightly (Kay & Phinney, 1956; Goodwin, 1956, unpublished work).

There was no indication in any of the autumn leaves examined of significant amounts of unknown oxidation products reported by Karrer & Walker (1934), although they included sycamore leaves in their study, nor of zeaxanthin which Strain (1938) found in fallen peach, poplar and catalpa leaves. The xanthophyll esters of Kuhn & Brockmann (1932) were encountered only in the sycamore; Kuhn & Brockmann found them to be widely distributed, although they did not examine the oak or black plum.

It is plain from these observations that no simple generalization can be made for the fate of xanthophylls in autumnal leaves, because significant differences were found to exist in the three trees examined in this study.

SUMMARY

1. The changes in the constituent chlorophylls and carotenoids of the leaves of three trees [the black plum (*Prunus nigra*), the oak (*Quercus robur*) and the sycamore (*Acer pseudoplatanus*)] have been followed from June to November. In *P. nigra* the anthocyanin was also examined.

2. In both the oak and the sycamore, the chlorophyll and carotenoid levels fell almost to zero, but whereas in the oak the levels fell simultaneously, in the sycamore the drop in chlorophylls preceded that in carotenoids. In the black plum the carotenoids tended to disappear first, but both carotenoid and chlorophyll levels fell only to about 50% of their starting values.

3. In all cases β-carotene and neoxanthin disappear more rapidly than do the other carotenoids, and lutein 5:6-epoxide appears in small amounts. In the sycamore but not in the other two trees, both lutein and violaxanthin esters appear in relatively large amounts in the later stages. These esters exist mainly in the cytoplasm and not in the chloroplasts.

4. In all cases the ratio chlorophyll *a*:*b* falls slightly during the late autumn.

5. There was no loss of anthocyanin in *P. nigra* leaves.

Thanks are due to Miss B. M. Eales for skilled technical assistance, to Dr E. C. Bate-Smith for information concerning the anthocyanins present in *Prunus* spp. and to Mr J. Duncan (University of Liverpool Botanic Gardens) for providing leaves of *P. pissardii*.

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The Labelling of Ram Semen *in vivo* with Radioactive Phosphate and [*carboxy*-^{14}C]Stearic Acid

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Isotopes have been used on a number of occasions to study the rates of physiological processes within the male reproductive tract. Howard & Pelc (1950), Risley (1955) and Pelc (1957) used radioautography to investigate spermatogenesis in the testes after the injection of [^{32}P]phosphate or [8-^{14}C]adenine. Ortavant (1954*a*, *b*) determined the time for ^{32}P-labelled deoxyribonucleic acid to appear in the sperm at the head of the epididymis and also observed the time taken for the labelling of various parts of the epididymis. Heath, Rimington, Glover, Mann & Leone (1953) and later Heath, Rimington & Mann (1957) found that when [^{35}S]methionine was injected into the boar, maximum activity appeared in the seminal-plasma proteins of serially collected samples of semen within a few days, whereas there was a delay of at least 3 weeks before the sperm proteins with maximum labelling were ejaculated. Similarly, Bronsch & Leidl (1957) have recently found that it took 14 days for maximum ^{32}P activity to appear in ejaculated bull semen after the administration of labelled phosphate, and Sirlin & Edwards (1955) found that it required 28 days to elapse before radioactive sperm were ejaculated from mice injected with [8-^{14}C]adenine.

The present experiments were started in the first instance to gain information about the functions and origin of glycerylphosphorylcholine in the reproductive tract of the ram, by studying the rate at which it appeared labelled in the semen after the administration of radioactive phosphate. This choline-containing phosphodiester was first reported as a constituent of the seminal vesicles of the rat (Diament, Kahane & Lévy, 1952; Lundquist, 1953) and more recently a survey has shown it to be a universal constituent of mammalian semen, where it often occurs in considerable concentration (Dawson, Mann & White, 1957). Analysis of the epididymal seminal plasma of various farm animals showed this to be a rich source of glycerylphosphorylcholine, and indicated that some of the diester was formed in such parts of the reproductive tract as the testes or epididymides rather than in the accessory glands, where many of the seminal-plasma components are usually secreted.

The present isotopic results are consistent with the view that most of the seminal glycerylphosphorylcholine is formed in the epididymides. As the diester is probably produced by the hydrolysis of lecithin, experiments were made to determine whether its production was associated with a transfer of labelled fatty acids or lipid to the sperm; no evidence for such a transfer was found.

These isotopic experiments have been extended to include observations upon the rate of appearance of radioactivity in various phosphorus components of sperm in serially collected samples of semen after administration of ^{32}P. From these results assessments have been made of the time taken for sperm to pass through the epididymides and also for certain stages of spermatogenesis to occur in the testes. These measurements possess some advantage over methods used previously in that, if it can be assumed that the tracer causes no radiological damage to the testes, the observations have been made directly upon the sperm under normal physiological conditions.

EXPERIMENTAL

Collection and fractionation of semen

Rams (Clun Forest) were trained to use an artificial vagina and ejaculates were collected every 2 or 3 days during the breeding season. Lengths of bicycle inner tubing (1¾ in. diam.) were found to make very durable liners for the vagina and lubrication was provided with petroleum jelly except in experiments involving labelled stearic acid, in which no lubrication was used. The temperature was maintained at 40–42°, a 12 v heating coil supplied through a mains transformer being used. After collection the sample of semen was stored at 37° or in a Thermos receiver. The volume was recorded, and small portions were used for motility determinations and sperm counts. The semen (0·5–1·4 ml.) was centrifuged and the seminal plasma (0·3–1·1 ml.) was removed with a teat pipette; the sperm were then washed four times with a calcium-free Ringer solution (Dawson *et al.* 1957) by centrifuging. In experiments requiring observations on the acid-soluble phosphorus of the sperm the last two washings were carried out with Ringer solution from which phosphate had been omitted.

Seminal plasma. The seminal plasma was introduced into 5 ml. of ethanol, and after mixing and standing for 5 min. the precipitate was removed by centrifuging. The supernatant was evaporated to dryness *in vacuo*, and the

residue was shaken with a mixture of 2 ml. of water and 3 ml. of a $CHCl_3$–butan-2-ol mixture (2:1, v/v). On centrifuging, the emulsion separated cleanly; the lower lipid–solvent layer contained insignificant amounts of lipid phosphorus. The upper aqueous layer, containing the glycerylphosphorylcholine, was evaporated to near dryness and applied as a short streak on a uni-dimensional filter-paper chromatogram. Development was carried out with a solvent which consisted of phenol saturated with water–ethanol (4:1, v/v); this enabled the diester, which ran almost with the solvent front, to be cleanly separated from the small amounts of other water-soluble phosphorus components of the seminal plasma present in the ethanolic extract. Location of the glycerylphosphorylcholine spots and their oxidation and solution before radioactive assay and phosphorus analyses have been described previously (Dawson, 1955).

Washed sperm. The washed sperm were treated with 10 ml. of ice-cold trichloroacetic acid soln. (10%). After standing at 0° for 10 min. the suspension was stirred and centrifuged. The supernatant was filtered and the radioactivity of the acid-soluble phosphorus was determined directly with a liquid counter, the phosphorus content being estimated in a 5 ml. portion of the solution. The precipitate was washed with 5 ml. of ice-cold water, and then treated with 10 ml. of a $CHCl_3$–methanol mixture (1:1, v/v) to extract the phospholipids. After standing for at least 1 hr., with occasional stirring, the mixture was centrifuged and the lipid extract was poured into a 100 ml. beaker containing 90 ml. of 0·01 M-$MgCl_2$ soln. After leaving for about 5–10 hr., the clear $CHCl_3$ lower layer containing the phospholipids was separated from the upper aqueous layer with a teat pipette. In this way any residual water-soluble phosphorus contaminating the phospholipids was eliminated (Folch, Ascoli, Lees, Meath & LeBaron, 1951). It was noticed that much cleaner interfaces of the $CHCl_3$ and aqueous layer could be obtained at this purification stage by drastically limiting the amount of petroleum jelly used to lubricate the artificial-vagina liner. The radioactivity of the lipid extract was estimated directly with a liquid counter after dilution to 10 ml. with ethanol. Phosphorus was determined in a 3 ml. portion after this had been evaporated to dryness and oxidized to inorganic phosphate.

The lipid-free sperm residue was washed with a further 10 ml. of the $CHCl_3$–methanol solvent, and then dried *in vacuo*. The residue was then incubated for 18 hr. at 37° with 3 ml. of N-NaOH soln., and after cooling to 0° the volume was brought up to 10 ml. by the addition of ice-cold 10% trichloroacetic acid soln. After standing for 10 min. at 0° the precipitated deoxyribonucleic acid was centrifuged off, washed with a further 10 ml. of the trichloroacetic acid soln., and then oxidized to inorganic phosphate. It was found that this could be very rapidly done by adding to it 0·05 ml. of 10% ammonium molybdate soln. and 1 ml. of 72% (w/w) $HClO_4$. On warming to 150° on an electrical digestion rack, shielded to avoid explosive risks, oxidation was very rapid. To avoid the formation of a water-insoluble precipitate the digestion mixture was removed from the rack immediately it turned pale yellow or became colourless. It was made up to 10 ml. with water and any turbidity removed by keeping it in a boiling-water bath until the precipitate dissolved. This solution, containing the deoxyribonucleic acid phosphorus, was assayed for radioactivity with a liquid counter, and phosphorus was determined in a 1 ml. portion. The supernatant from the precipitation of the deoxyribonucleic acid (the ribonucleic acid + phosphoprotein phosphorus fraction of Schmidt & Thannhauser, 1945) was also assayed for radioactivity and phosphorus. Its specific radioactivity seemed to be distinct from that of the other phosphorus-containing fractions of the sperm, and, although its chemical composition is uncertain and undoubtedly complex, it is for convenience designated as the 'residual phosphorus' fraction in this paper.

In some experiments the acid-soluble phosphorus of the sperm was not examined and here the Ringer-washed sperm were directly extracted with $CHCl_3$–methanol solvent to remove the lipids.

In the experiments involving sperm doubly labelled with [*carboxy*-^{14}C]stearic acid and [^{32}P]phosphate the collection and fractionation procedure was modified as follows. No lubrication was used on the liner of the artificial vagina, in order to avoid contamination of the semen with lubricant. The fatty acids and phosphorus in the seminal-plasma lipids were separated for radioactive assay in the following way. The $CHCl_3$–butan-2-ol-soluble substances present in the ethanolic extract of seminal plasma were recovered by removing the solvent *in vacuo*. The residue was then hydrolysed for 16 hr. at 100° with 2 ml. of 5N-HCl. The fatty acids were extracted with 8 ml. of ether, and the ether solution was evaporated and eventually dried on a planchet in a desiccator ready for weighing and radioactive assay. The HCl hydrolysate was evaporated to dryness and oxidized; it was found to contain only negligible amounts of phosphorus and its radioactivity was not examined further. This process of separating the phospholipid phosphorus and lipid fatty acids was also repeated for the sperm lipids after they had been extracted and purified as described beforehand.

Administration and assay of isotopes, etc.

Labelled phosphate was administered with carrier (0·5 mg. of ^{31}P) in 5 ml. of isotonic NaCl soln. via the jugular vein. The dose used was approximately 1 mc/10 kg. At this level the animals showed no sign of radiation sickness, and the motility and morphological form of the ejaculated sperm appeared normal, so it is unlikely that any damage had been caused to the testes. [*carboxy*-^{14}C]Stearic acid (1 mc, 13 mg.) was also given via the jugular vein as a colloidal suspension in isotonic NaCl soln. Radioactivity of ^{32}P was measured in an M6 liquid counter and ^{14}C with a mica end-window counter. Specific radioactivity of the fatty acids was expressed as counts/mg., which was sufficiently accurate for the present experiments, and of the ^{32}P as counts/mg. of phosphorus. Decay corrections were applied to the ^{32}P observations. Phosphorus was determined by the method of Fiske & Subbarow (1925).

RESULTS

In initial experiments washed ram sperm were suspended in a Ringer solution containing labelled phosphate and fructose and incubated aerobically in Warburg vessels. No significant labelling of the sperm deoxyribonucleic acid or phospholipids occurred in 4 hr. even though respiration was

active. This provided a basis for performing experiments *in vivo*, for it seemed likely that in the reproductive tract the main labelling of these phosphorus components of the sperm would occur on their formation rather than by a continual dynamic exchange as is seen with tissue components. Immediately after the injection of labelled phosphate into a ram the specific radioactivity of the acid-soluble phosphorus in the blood plasma is very high, but its level then rapidly falls as the phosphate equilibrates with the phosphate of the bones and other tissues. Eventually the rate of decline levels out until a very slow and more or less constant decline is observed, which corresponds to the excretion of active phosphate by the animal and its replacement with the inactive phosphorus of the diet (Fig 1).

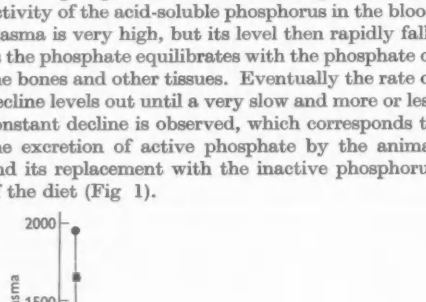

Fig. 1. The decline of radioactivity in the acid-soluble fraction of ram-blood plasma after the intravenous injection of labelled phosphate. The ram (wt. 94·6 kg.) was injected with 0·8 mc of [^{32}P]phosphate.

Isotope distribution in normal semen

When serial samples of semen were collected from rams which were ejaculating motile spermatozoa it was found that the various phosphorus fractions of the ejaculated semen became labelled in a definite sequence which was constant from animal to animal. In these experiments ejaculates were collected from the rams about two or three times a week, and it is probable that if the frequency of collection was varied the rate of appearance of labelled phosphorus components would also change. Fig. 2 shows a typical result obtained from the present experiments. At first, within a week of the administration of isotope to the animal, the acid-soluble phosphorus and the 'residual phosphorus' of the sperm acquired a small amount of labelling, but as collections were continued this declined again to a low level. From other observations to be described later it seems likely that this may be due to the initial secretion of very high specific-radioactivity phosphate by the accessory glands or contamination of the semen with urine phosphate of high activity. This highly radioactive phosphate in the seminal plasma might then enter or adhere to the sperm and introduce activity into the acid-soluble phosphorus fraction or exchange with the 'residual phosphorus' of the sperm. Then on the rapid decline of the specific activity of the blood-plasma phosphate the magnitude of this type of exchange labelling is likely to fall to a very low level in subsequent semen samples. Such an exchange of inorganic phosphorus has been observed with bull spermatozoa *in vitro*; when these were suspended in a saline containing labelled phosphate, radioactive ionic equilibrium was established within 5–45 min. (Bishop & Weinstock, 1948).

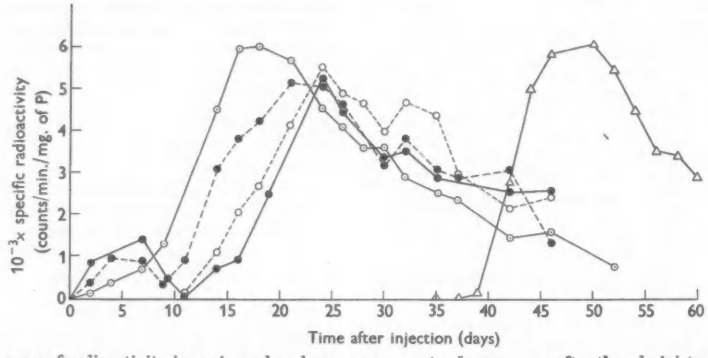

Fig. 2. Appearance of radioactivity in various phosphorus components of ram semen after the administration of labelled phosphate. ⊙—⊙, Seminal-plasma glycerylphosphorylcholine; ○- - -○, sperm phospholipid; ●- - -●, sperm acid-soluble phosphorus; △—△, sperm deoxyribonucleic acid; ●—●, sperm 'residual phosphorus.' The ram (wt. 41·5 kg.) was injected with 5 mc of [^{32}P]phosphate.

The labelling of the seminal-plasma glycerylphosphorylcholine began at a very low level in samples of semen collected a few days after the injection, but did not reach a maximum until about $2\frac{1}{2}$ weeks later (Fig. 2). In four animals ejaculating motile sperm the maximum labelling of the glycerylphosphorylcholine occurred in ejaculates collected at 17, 18, 15 and 15 days after injection and always well before the phosphorus-containing components of sperm became maximally labelled. The maximum labelling of the sperm phospholipids occurred in four rams ejaculating motile sperm in ejaculates collected at 24, 21, 23 and 26 days after the isotope injection. Only one of these animals was examined for sperm acid-soluble phosphorus and 'residual phosphorus', and here as well as in another ram ejaculating non-motile sperm maximum labelling of these fractions occurred at about the same time as the sperm phospholipids.

No labelled sperm deoxyribonucleic acid appeared in the ejaculates until about 40–45 days after the administration of isotope: its specific radioactivity then increased with each successive ejaculation until a maximum was reached at 50 and 52 days in the two normal animals examined. Owing to the comparatively large amount of deoxyribonucleic acid in semen an accurate assessment of its specific radioactivity can be made after this length of time even though the half-life of ^{32}P is only 14·3 days. With other phosphorus components of the semen this is more difficult because of their lower total concentration, but nevertheless such readings as have been obtained gave no evidence of specific-radioactivity peaks corresponding with that of the deoxyribonucleic acid.

Isotope distribution in semen from a ram ejaculating non-motile sperm

In a single animal which normally ejaculated motile sperm, a transient period was experienced when the sperm ejaculated were non-motile but present at the same density as normally. Although the cause of this temporary defect could not be ascertained, isotope was administered during this period and a study made of the labelling of samples of semen collected serially. In nearly every respect this was similar to results obtained with normal semen except that the appearance of labelled glycerylphosphorylcholine was delayed (Fig. 3). Thus whereas in the four normal animals maximum labelling of the diester appeared at 15–18 days, and well before that of the sperm phospholipids, in the defective ram the maximum occurred at 30 days and almost simultaneously with the sperm phospholipids. While no general conclusions can be reached from this observation on a single ram it would appear that in this particular animal the secretion of radioactive glycerylphosphorylcholine in the reproductive tract or its passage to the exterior was considerably altered or delayed, although its concentration in the semen was normal. It is hoped that future opportunities may occur to confirm this finding with other animals.

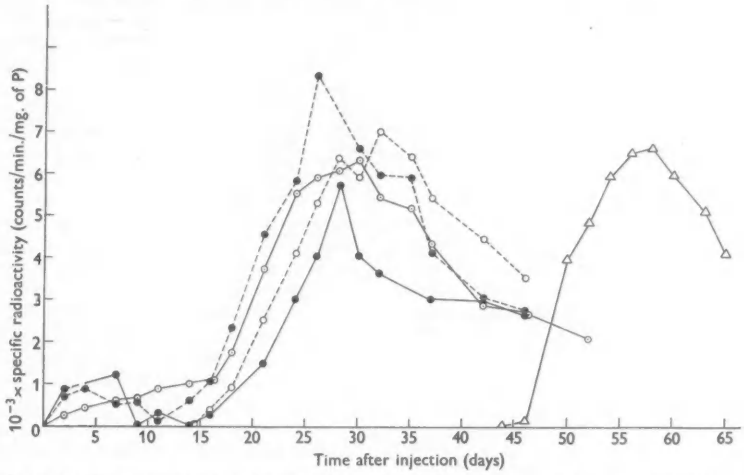

Fig. 3. Appearance of ^{32}P-radioactivity in various phosphorus components of semen in a ram producing non-motile sperm. ⊙—⊙, Seminal-plasma glycerylphosphorylcholine; ○- - -○, sperm phospholipid; ●- - -●, sperm acid-soluble phosphorus; △—△, sperm deoxyribonucleic acid; ●—●, sperm residual phosphorus. The ram (wt. 65·5 kg.) was injected with 7·5 mc of [^{32}P]phosphate.

Effect of severing the connexion between testes and epididymides

In order to obtain more information about the ^{32}P-labelling of semen other than in the testes, in one animal the connexions between the testes and epididymides were surgically severed. On one side the entire testis was removed and the epididymis with its blood supply was left intact. On the other side the testis was left *in situ*, to supply testosterone, and the head of the epididymis was removed. Three hours after the operation the animal was injected with [^{32}P]phosphate and serial samples of semen were then collected as usual. Considerable swelling of the scrotum occurred initially, and slight fever, but nevertheless collections of semen every 2–3 days were successfully carried out. Fig. 4 shows the total sperm present in the ejaculated semen, from which it can be concluded that it took approx. 15 days for sperm to be virtually cleared from the epididymis. For the first three collections the sperm were motile but then, possibly because of the transient slight fever of the animal, they became progressively less motile. The sperm phosphorus-containing components showed little labelling except in the acid-soluble phosphorus fraction. Part of this labelling may be due to the presence of active glycerylphosphorylcholine in or on the sperm, as they are known to contain small quantities of the diester which is not removed by washing; some may also arise from an exchange with highly active accessory-gland secretion or urine phosphate after ejaculation. No significant labelling of sperm phospholipids, deoxyribonucleic acid or 'residual phosphorus' occurred in these sperm obtained from the epididymides.

The decrease in the number of sperm ejaculated was associated with a fall in the concentration of glycerylphosphorylcholine. Nevertheless the specific-radioactivity curve of the diester was very similar to the normal pattern, which would suggest that part at least of the diester has an extratesticular origin.

Labelling of semen after simultaneous administration of [^{32}P]*phosphate and* [carboxy-^{14}C]*stearic acid*

In order to find whether any correlation existed between the formation of glycerylphosphorylcholine and the labelling of the fatty acids present in semen, an experiment was performed in which a ram was injected simultaneously with both labelled phosphate and [*carboxy*-^{14}C]stearic acid. The appearance of labelling of the phosphorus-containing components of the ejaculated semen followed a course which was typical (Fig. 5). The first ^{14}C-labelling of the sperm fatty acids appeared in the ejaculate soon after injection, i.e. within

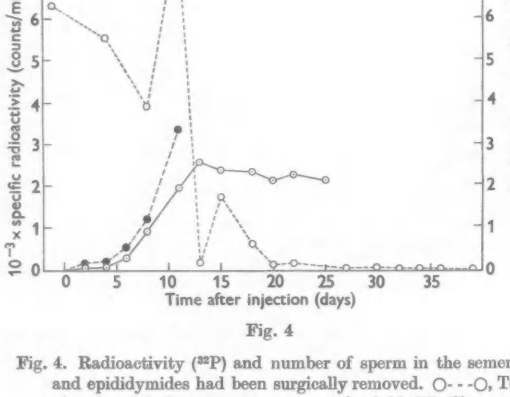

Fig. 4

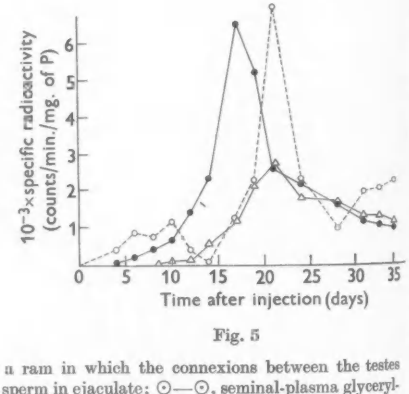

Fig. 5

Fig. 4. Radioactivity (^{32}P) and number of sperm in the semen of a ram in which the connexions between the testes and epididymides had been surgically removed. ○- - -○, Total sperm in ejaculate; ⊙—⊙, seminal-plasma glycerylphosphorylcholine; ●- - -●, sperm acid-soluble ^{32}P. The ram (wt. 74 kg.) was injected with 2·7 mc of [^{32}P]phosphate.

Fig. 5. Specific radioactivity of the semen glycerylphosphorylcholine (^{32}P), phospholipids (^{32}P) and sperm fatty acids (^{14}C) after the injection of labelled phosphate and [*carboxy*-^{14}C]stearic acid. ●—●, Seminal-plasma glycerylphosphorylcholine (^{32}P); △—△, sperm phospholipid (^{32}P); ○- - -○, sperm fatty acid (^{14}C). The ram (wt. 56·6 kg.) was injected with 3·5 mc of [^{32}P]phosphate and 1 mc of [*carboxy*-^{14}C]stearic acid.

10 days, and this suggests an accessory-gland origin. In this connexion maximum ^{14}C-labelling of the seminal-plasma fatty acids was found to occur soon after the injection of the isotope and Bishop & Lovelock have recently obtained evidence that isolated bull sperm can incorporate added [*methyl*-^{14}C]acetate into their lipid fatty acids (Austin & Bishop, 1957 and personal communication). There was no peak in the sperm fatty acids corresponding to the time when maximum ^{32}P-activity was found in the ejaculated glycerylphosphorylcholine, but as is to be expected a distinct ^{14}C peak appears in them at the time of maximum labelling of the sperm phospholipids.

DISCUSSION

It has been found that when a ram is injected with labelled phosphate the specific radioactivity of the acid-soluble phosphorus in the blood plasma rapidly declines as the isotope equilibrates with the phosphorus in the tissues. While there is some evidence that the passage of phosphate ions from the blood plasma into the testes may be slower than with other organs (Hevesy & Ottesen, 1943), it is to be expected that once inside the tissues of the reproductive tract the labelled phosphorus atoms will rapidly be incorporated into the metabolism which is responsible for the synthesis of the various phosphorus-containing components of the sperm and seminal plasma. This will mean that comparatively soon after the injection the various phosphorus-containing components used in the formation of the sperm and seminal plasma will appear in the reproductive tract in a radioactive form. Thus any delay in the appearance of the labelled component in the ejaculated semen must largely represent the time taken by various physiological processes in the reproductive tract before that component is ejaculated. Such processes would include, for example, the completion of spermatogenesis or the passage of sperm through the much convoluted tubule of the epididymis. This argument would only be valid if no appreciable exchange of the phosphorus in the seminal phosphorus compounds occurs after their formation. Experiments *in vitro* have indicated that this is probably true for the structural elements, e.g. phospholipids and deoxyribonucleic acid of ejaculated sperm, but it is apparent that some exchange of the acid-soluble phosphorus and 'residual phosphorus' fractions does occur, possibly by a passage of high-activity phosphate into the sperm from the seminal plasma after ejaculation.

One experimental point which has been clearly established in the present work is that, in the ram ejaculating motile sperm, labelled glycerylphosphorylcholine appears in the semen appreciably before the phosphorus components of the sperm become labelled. Previous work has indicated that in many animals glycerylphosphorylcholine is present in the epididymal seminal plasma at a considerable concentration (Dawson *et al.* 1957), which would suggest that it is formed either in the testis or epididymis. If then the glycerylphosphorylcholine which appears as a component of the plasma in ejaculated semen moves through the epididymis at an equal or slower rate to that of the sperm the present results would indicate that a considerable formation of the phosphodiester occurs in the epididymis. This is supported by the observation that considerable ^{32}P-labelling of seminal-plasma glycerylphosphorylcholine occurred in an animal surgically treated to sever the connexion between the testis and epididymis. However, a puzzling anomaly does remain: in one animal ejaculating non-motile sperm the labelling of the glycerylphosphorylcholine of the semen appeared at a point which exactly corresponded to the labelling of the phosphorus component of the sperm apart from deoxyribonucleic acid. This cannot be due to any reduction in the rate of secretion of glycerylphosphorylcholine into the semen because the amounts ejaculated by the animal were normal.

No explanation has yet been found for the considerable quantities of glycerylphosphorylcholine found in epididymal seminal plasma. So far the only metabolic pathway shown for the formation of glycerylphosphorylcholine in mammalian tissues appears to be by the hydrolysis of lecithin (e.g. Dawson, 1955). One possibility would be that in the reproductive tract lecithin is broken down in this way, and that the fatty acids are donated to the sperm in the form of some lipid, the glycerylphosphorylcholine formed in the process passing out as a by-product. In this connexion it was suggested by Redenz (1924) from histological observations that the epididymis was responsible for the formation of the lipid capsule of the sperm. However, in an experiment in which labelled stearic acid was administered simultaneously with labelled phosphate to a ram, no evidence of a transfer of this type could be detected; at the time of the appearance of ^{32}P-labelled glycerylphosphorylcholine in the semen no equivalent ^{14}C-labelling peak was observed in the fatty acids of the sperm. On the other hand, as is to be expected, an equivalent ^{14}C peak was clearly observed at the time of the maximum ^{32}P-labelling of the sperm phospholipids.

Another point which has been established is that after injection of [^{32}P]phosphate in the ram, the deoxyribonucleic acid of the sperm appears labelled in serially collected ejaculates at a con-

siderable time after the other phosphorus components of the sperm are ejaculated in a radioactive form. One explanation of this would be that the other phosphorus components become labelled during the passage of the sperm down the reproductive tract some weeks after they have left the testes. Ortavant (1954*a*), for example, found some evidence for an exchange of ^{32}P of this type at the head of the epididymis. However, from histological observations it is apparent that the sperm is virtually morphologically complete as it leaves the testes and one would expect the maximum labelling of, for example, the sperm phospholipids to occur during the formation of the sperm structure in the testes. Moreover, when the passage of the sperm from the testes was suppressed surgically the sperm in the epididymis did not become significantly labelled with ^{32}P except in their acid-soluble phosphorus fraction. If then the phosphorus components of the sperm become labelled mainly in the testes this must mean that either labelled deoxyribonucleic acid for sperm construction is formed considerably after, for example, labelled phospholipid, or alternatively that both are formed at the same time and that the nucleic acid is used in the formation of the sperm at a time during spermatogenesis well before that of the phospholipids. The whole concept of dynamic turnover of tissue components is against the first idea, for however enormous the pool size of the precursor of sperm deoxyribonucleic acid one would expect some labelling of this to occur even though it might be small compared with that of the phospholipids. In rats, for example, Hevesy & Ottesen (1943) found that at least 2·6% of the testicular deoxyribonucleic acid was replaced daily by newly synthesized material, and Pelc (1957) found that up to 12% of the spermatogonia possessed labelled deoxyribonucleic acid in their nuclei 1 day after the injection of [8-^{14}C]adenine. It seems much more likely therefore that deoxyribonucleic acid is formed during spermatogenesis only during the mitotic divisions taking place during the formation of the spermatocytes, whereas it is likely that phospholipid would be added up to the completion of the finished sperm. Evidence is accumulating from spectrophotometric examination of cells that the primary spermatocyte contains all the deoxyribonucleic acid necessary for the formation of the nuclei of the four spermatozoa which it finally produces (Lison & Pasteels, 1949; Swift, 1950; Schrader & Leuchtenberger, 1950). Furthermore, experiments with [8-^{14}C]adenine in the mouse testis have shown heavy incorporation into the deoxyribonucleic acid of non-dividing spermatogonia and primary spermatocytes, a small incorporation into secondary spermatocytes and a very small incorporation in the later stages of spermatogenesis (Pelc, 1957). If the production of spermatozoa is a continual process this would mean that sperm with labelled phospholipids would pass from the testis some time before those with labelled deoxyribonucleic acid. The time elapsing therefore between the first appearance of labelled phospholipid in the sperm of the ejaculate and labelled deoxyribonucleic acid (25–35 days) would represent the minimum time which it takes for the primary spermatocyte to be converted into the spermatozoa during spermatogenesis. In experiments with rams injected with [^{32}P]phosphate Ortavant (1954*b*) found that it took approximately 30 days for sperm labelled in their deoxyribonucleic acid fraction to appear at the head of the epididymis. This time therefore agrees well with the interpretation placed on the present results.

The present experimental evidence suggests that no appreciable ^{32}P exchange of sperm phospholipids occurs after they have left the testes. If this is so the results can be used to derive the minimum time necessary for spermatozoa to pass through the epididymides. Thus in four animals which were ejaculating naturally three times a week it took 13, 14, 10 and 13 days for labelled phospholipids to appear in the sperm. It is clear, however, that the rate of passage of sperm through the epididymis will depend to some extent upon the frequency of ejaculation and other biological factors. Thus by killing, at various intervals, rams which had been injected with [^{32}P]phosphate and examining the epididymides for activity, Ortavant (1954*a*) calculated that it took 20 days for sperm to pass through the epididymides in animals not sexually active.

The advantage of using isotopic labelling of the sperm to make these measurements is that provided no radiation damage is done to the testes, the movement of the sperm is assessed directly under normal physiological conditions. Previous estimates have usually relied on the injection of indian-ink particles into the testes, and it is apparent that such foreign bodies may not move at the same rate as the sperm themselves. Gunn (1936) found that when indian-ink particles were injected into the testes of rams it took 11 days in sexually resting rams, and 5 days in animals frequently caused to ejaculate by electrical stimulation, for the particles to pass through the reproductive tract. On the other hand, McKenzie & Phillips (1934) found that after scrotal insulation to cause heat degeneration of the testes it took an average of 8·8 days (4–13) for abnormal sperm to pass through the genital passages of rams copulating at the rate of approximately once a day. In arriving at this result they assume that no morphological change is brought about in the sperm by the heat after the completion of spermatogenesis.

SUMMARY

1. Rams were injected with [^{32}P]phosphate and [*carboxy*-^{14}C]stearic acid, and a study was made of the rate of appearance of labelled phosphorus compounds and fatty acids in the seminal plasma and sperm of serially collected samples of semen.

2. The maximum ^{32}P-labelling of seminal glycerylphosphorylcholine occurred some 15–18 days after injection, and next appeared the sperm phospholipids, acid-soluble phosphorus and a fraction designated as residual phosphorus, all having maxima at about 21–26 days. The sperm deoxyribonucleic acid appeared in a labelled form much later, reaching a maximum at 50–52 days.

3. In one animal ejaculating non-motile sperm the appearance of labelled glycerylphosphorylcholine in the semen was considerably delayed compared with normal rams.

4. In a ram in which the connexions between the epididymides and testes were severed surgically the seminal-plasma glycerylphosphorylcholine and sperm acid-soluble phosphorus became labelled with ^{32}P, but no appreciable activity appeared in the phospholipids, residual phosphorus and deoxyribonucleic acids of the ejaculated sperm.

5. Simultaneous injection of a ram with [^{32}P]-phosphate and [^{14}C]stearic acid did not indicate any transfer of labelled fatty acids to the sperm at the time of formation of glycerylphosphorylcholine.

6. From these results with isotopes an assessment has been made of the minimum time taken for the sperm to pass through the epididymides and also the time taken for the spermatocytes to be converted into the spermatozoa during spermatogenesis in the ram testes.

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Comparative Studies on the Liver Sulphatases

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Although the widespread distribution of the arylsulphatases is well known, there is little information available on the amounts of these enzymes present in the various species, the only attempt at comparative studies being that of Rutenburg & Seligman (1956) whose work is diminished in value because of the failure to appreciate the occurrence of several different sulphatases in their preparations. At least three arylsulphatases occur in mammalian tissues; sulphatases *A* and *B* (Roy, 1953*b*, 1954*a*) rapidly hydrolyse dipotassium 2-hydroxy-5-nitrophenyl sulphate (nitrocatechol sulphate, NCS) but attack potassium *p*-nitrophenyl sulphate (NPS) only very slowly. These two enzymes may be separated by paper electrophoresis at pH 7, sulphatase *A* moving towards the anode

and sulphatase *B* towards the cathode. Sulphatase *C*, in striking contrast, hydrolyses NPS very rapidly and unlike the former two enzymes is extremely difficult to obtain in true solution (Roy, 1956*c*). The assay of the arylsulphatases in crude tissue preparations is further complicated by a number of factors, including the following: the destruction of the liberated phenol by the tissue (Dodgson & Spencer, 1953), the occurrence of an endogenous inhibitor in many tissues (Maengwyn-Davies & Friedenwald, 1954), the influence of many naturally occurring ions on the activities of the arylsulphatases (Roy, 1955, 1956*c*), the insolubility of sulphatase *C* (Roy, 1956*c*) and the highly anomalous kinetics of sulphatase *A* (Roy, 1953*b*). These factors make the assay of the arylsulphatases in unfractionated tissue preparations a matter of some difficulty.

A method has, however, been developed for the assay of the arylsulphatases in liver preparations. It depends upon the isolation of the usual cell fractions from a homogenate of the tissue, followed by the assay of each fraction for the various enzymes. The sum of these activities is taken as being the activity of the whole tissue. By this means many of the complicating factors listed above could be eliminated, or at least their effects made relatively constant. As already demonstrated (Roy, 1954*b*), isolated cell components do not destroy nitrocatechol; the concentration of sulphatase *C* in the microsomal fraction (Dodgson, Spencer & Thomas, 1954) and of sulphatases *A* and *B* in the mitochondrial and microsomal fractions (Roy, 1954*b*) allows the use of short incubation times, which in turn eliminates many of the complex kinetics shown by sulphatase *A* (Roy, 1957*b*). The preparation of the cell fractions under highly standardized conditions minimizes the effects of any endogenous inhibitors which may be present. Those occurring in the soluble fraction of the cell will accumulate in the final supernatant, which contains little sulphatase activity, and the amounts of those occurring in any of the particulate fractions will be kept constant by the standardized method of preparation.

This method has been applied to a study of the distribution of the arylsulphatases in the livers of various species and of changes in the arylsulphatase activity brought about by a number of experimental procedures. Determinations of steroid sulphatase activity (Roy, 1957*a*) and of the ability to synthesize dehydro*epi*androsterone sulphate (DHAS) (Roy, 1956*b*) were carried out at the same time.

EXPERIMENTAL

Preparation of nitrocatechol sulphate. The method used was that of Roy (1953*a*) with modifications similar to those of Dodgson & Spencer (1956) to remove the small amounts of nitropyrogallol disulphate which contaminated the original preparations (Roy & Kerr, 1956). The method is given here in detail because of the increasing use of NCS as a substrate for the assay of the arylsulphatases.

To 350 g. of KOH and 350 g. of potassium persulphate in 5 l. of water was added 150 g. of *p*-nitrophenol, and the mixture was stored at 37° for 48 hr. After bringing to pH 4 with H_2SO_4 the mixture was cooled to 5° and filtered, the precipitate being discarded. Phenols remaining in the filtrate were removed by extracting five times with 1 l. portions of ether. The aqueous phase was brought to pH 10–11 with KOH soln., left overnight at 5°, decanted from the underlying crystalline mass and concentrated *in vacuo* to about 750 ml. Acetone (2 vol.) was added and the precipitate of inorganic material filtered off. The filtrate was concentrated *in vacuo* until crystallization occurred. When crystallization was complete the crude NCS, as the potassium phenoxide, was filtered off and sucked dry at the pump. The filter cake was dissolved in boiling water and acetic acid added (1 ml./g. of filter cake) to precipitate the NCS in the phenolic form. This was twice recrystallized from water to remove the nitropyrogallol disulphate and then reconverted into the potassium phenoxide by crystallizing from the minimum volume of boiling water containing a 20% excess over the theoretical amount of KOH. The NCS was finally recrystallized from water. The usual yield is approximately 15 g. and another 4 g. may be obtained from the mother liquors of the last two crystallizations.

The particular crystalline form which is obtained depends upon the exact conditions of the crystallization. Rapid cooling and constant agitation of the hot saturated solution causes a monohydrate to appear as yellow needles from the still warm solution. Slow cooling without agitation causes orange prisms of a dihydrate to crystallize from the cold solution. Drying over P_2O_5 *in vacuo* at room temperature converts the dihydrate into the monohydrate and finally into the anhydrous form. The drying is slow and rather erratic, so that the anhydrous compound is best obtained by finally drying the material over P_2O_5 *in vacuo* at 60° for 4 hr. (Found: C, 23·1; H, 1·1. Calc. for $C_6H_3O_7NSK_2$: C, 23·1; H, 0·97%).

It should be noted that NCS, as the potassium phenoxide, explodes on heating, as do many simple phenoxides (Dr J. W. Minnis, personal communication). This behaviour necessitates the use of considerable care during elementary analyses.

Animal experiments. The animals used were stock animals from the various University departments. Partial hepatectomy in rats and mice was performed by the technique of Higgins & Anderson (1931). Necrosis of the liver in these species was induced by the subcutaneous injection of CCl_4 (5·3 g./kg.) as a 50% solution in olive oil (Levvy, Kerr & Campbell, 1948). In guinea pigs, scurvy was induced by feeding the ascorbic acid-deficient diet of Kodicek & Loewi (1955). Granuloma formation in guinea pigs was caused by the subcutaneous injection of a 1% solution of carrageenin (Robertson & Schwartz, 1953).

Cell fractionation. This was carried out by the standard technique of Hogeboom (1955), a 10% suspension of the liver in 0·25 M-sucrose being used as the starting material. The centrifugal forces used for separating the various cell fractions were: nuclei, 700 *g*; mitochondria, 5000 *g*; microsomes, 24 000 *g*. For assay, these fractions were sus-

pended in water to give a concentration corresponding to a 20% suspension of the liver and this was diluted with water as necessary (Table 1).

Enzyme assays. Sulphatases *A* and *B* together (sulphatase *A* + *B*) were determined with NCS as substrate in 0·13 M-acetate buffers. The general experimental procedures were as previously described (Roy, 1953*a*) and the exact conditions used for each species are summarized in Table 1. Sulphatase *C* was determined with NPS as substrate in 0·13 M-2-amino-2-hydroxymethylpropane-1:3-diol–acetic acid buffers (tris buffers). The general experimental conditions have already been described (Roy, 1956*c*) and the details are given in Table 1.

Steroid sulphatase was determined, as previously described (Roy, 1957*a*), with DHAS as substrate at pH 7·8 in tris buffer. Because of the small amounts of this enzyme present the optimum conditions for each of the species studied were not determined. Only the microsomal fraction was assayed as, at least in the rat (Roy, 1957*a*), steroid sulphatase activity is restricted to this fraction. The time of incubation was 3 hr. for rat and ox tissue; for all the other species incubation times of 17 hr. were required.

The ability to synthesize DHAS was determined, exactly as previously described (Roy, 1956*b*), with a second sample of liver (5 g.) to prepare the enzyme system. No attempt was made to determine the optimum conditions for the various species.

Paper electrophoresis. A 25% homogenate of the liver in water was prepared in a Potter–Elvehjem (1936) homogenizer and was centrifuged for 2 hr. at 24 000 *g* at 0°. A sample of the clear supernatant was removed, care being taken to avoid contamination both by the loosely packed sediment and by the upper layer of fatty material. Samples (0·1 ml.) of this material were then submitted to paper electrophoresis on Whatman no. 100 paper for 16 hr. at 200 v in 0·03 M-diethylbarbiturate buffer, pH 7·4. The paper was then cut into 1 cm. strips, which were incubated for an appropriate time (1–6 hr.) with nitrocatechol sulphate in acetate buffer. For sulphatases of the *A* type the conditions were 0·003 M-NCS at pH 5 in 0·13 M-acetate, and for the *B* type 0·03 M-NCS at pH 6 in 0·13 M-acetate. The amounts of nitrocatechol liberated were determined as usual (Roy, 1953*a*).

RESULTS

Fig. 1 shows that under the experimental conditions specified in Table 1 the amount of the phenol liberated is directly proportional to the concentration of the tissue in the reaction mixture.

Because of the complexity of the method a high degree of accuracy was not to be expected, but the results shown in Table 2 demonstrate it to be sufficiently reproducible for the present investigation. Similar experiments showed the validity of the method used in the assay of DHAS synthesis.

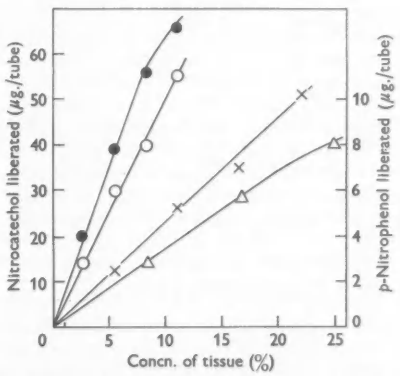

Fig. 1. Effect of tissue concentration on the rate of hydrolysis of sulphate esters. Volume of reaction mixture, 0·8 ml., containing 0·2 ml. of a suspension of the appropriate cell fraction equivalent to the concentration of whole tissue indicated. Substrate concentration and pH were as indicated in Table 1. Hydrolysis of NCS by rat-liver mitochondria, ○; by rat-liver microsomes, ●; by guinea-pig liver mitochondria, △. Hydrolysis of NPS by rat-liver microsomes, ×.

Table 1. *Conditions used in the assay of arylsulphatases in the livers of different species*

Volume of the reaction mixture was 0·8 ml., containing 0·2 ml. of tissue suspension corresponding to a 20% suspension of the liver except where otherwise specified. Incubation was for the time indicated.

	Sulphatase *A* + *B*			Sulphatase *C*		
Species	pH	Substrate concn. (mM)	Time (min.)	pH	Substrate concn. (mM)	Time (min.)
Rat	6·0*	30	15	7·7	4	15
Mouse	6·4†	30	15	8·3	5	60
Guinea pig	5·5	3	15	—	—	—
Rabbit	6·2	30	15	8·1	10	60
Ox	6·0	30	15	8·0	10	30
Hen	6·0	30	15	7·8	3	60
Frog	6·4†	30	15	—	—	—
Stickleback‡	6·0	30	15	—	—	—

* Tissue concentration: 5%. † Tissue concentration: 10%. ‡ Optimum conditions were not determined.

Comparative studies

The results of these experiments are summarized in Table 3. It should be noted that the units used to express the activity of steroid sulphatase and of the DHAS-synthesizing system differ from those used for the arylsulphatases by a factor of 10^{-3}.

Rat. The animals were of the Wistar strain, aged 3½–4 months. The most interesting result is the very pronounced sex difference in the activities of several of the enzymes. The sulphatase *C* and steroid sulphatase activities of the livers of male rats are about double those of the livers of female animals. These differences were highly significant ($P<0.001$). There was apparently a greater sulphatase $A+B$ activity in the livers of male rats, but this was not highly significant ($P<0.1$). As previously reported (Roy, 1956*b*), the ability to synthesize DHAS is very much greater in the livers of female rats than of male rats; this difference is again highly significant ($P<0.001$). Gonadectomy had little effect on these sex differences; even 8 weeks after the operation the only significant change was the fall in steroid sulphatase activity of the livers of castrated male animals.

In young rats, aged 2½ weeks, the sulphatase $A+B$ activity was extremely high; both sulphatase *C* and steroid sulphatase activities were slightly lower than normal.

Table 2. *Results of replicate analyses performed on single rat livers*

Samples 1–4 were obtained by treating independently four portions of one liver as described in the text. Samples 5–8 were obtained by fractionating four samples of a single homogenate of another liver. Results are expressed as μmoles of the phenol liberated/hr./g. of tissue under the conditions specified in Table 1.

Sample no.	Sulphatases $A+B$	Sulphatase C
1	195	19·6
2	211	19·2
3	248	21·5
4	196	21·7
Mean	213	20·5
5	195	18·4
6	200	23·4
7	186	21·7
8	193	21·0
Mean	194	21·1

Table 3. *Amounts of certain enzymes present in the livers of various species under different conditions*

Assays were carried out under the conditions listed in Table 1 and the results are expressed as μmoles of sulphate ester hydrolysed (the arylsulphatases), μm-moles of ester hydrolysed (steroid sulphatase) or synthesized (DHAS synthesis)/hr./g. of tissue in each case. Values quoted are the means, together with the standard deviations; the numbers of animals used are given in parentheses and details of the various treatments are given in the text. M, male; F, female; J, immature animals.

Species	Sex	Treatment	Sulphatases $A+B$	Sulphatases C	Sulphatases Steroid	DHAS synthesis
Rat	M	—	207±21 (8)	15·4±4·2 (8)	403±91 (8)	53±22 (20)
	M	Castration	208 (4)	12·1 (4)	279 (4)	72 (4)
	M	CCl_4	214±32 (8)	12·0±2·5 (8)	258±103 (8)	25±17 (8)
	F	—	187±20 (8)	8·0±1·2 (8)	187±62 (8)	149±37 (14)
	F	Castration	176 (4)	7·6 (4)	182 (4)	152 (4)
	F	Hepatectomy	297±22 (7)	8·9±2·0 (7)	177±74 (7)	135±25 (7)
	J	—	422 (4)	4·5 (4)	66 (2)	—
Mouse	M	—	55±14 (8)	1·5±0·4 (8)	64±11 (8)	7±4 (8)
	M	Hepatectomy	94 (4)	1·6 (4)	68 (4)	—
	F	—	35±16 (8)	1·6±0·4 (8)	52±19 (8)	34±18 (8)
	F	CCl_4	70±23 (8)	0·7±0·2 (8)	0 (8)	33 (3)
Guinea pig	M	—	52±12 (6)	0	0	72±9 (8)
	M	*Salmonella*	89±21 (5)	0	0	72±7 (5)
	M	Carrageenin	45 (4)	—	—	77 (4)
	M	Scorbutic animal	87 (4)	—	—	54 (4)
	F	—	38±7 (9)	0	0	78±11 (9)
	F	*Salmonella*	92±24 (6)	0	0	67±27 (6)
	F	Scorbutic animal	71 (2)	—	—	89 (2)
Rabbit	M	—	45 (2)	1·4 (2)	130 (1)	17 (1)
	F	—	61 (1)	1·7 (1)	106 (1)	18 (1)
Ox	M	—	29 (2)	15 (2)	300 (2)	19 (2)
Hen	F	—	26 (2)	2·3 (2)	†	8 (2)
Frog	M	—	160 (7)	0	0	†
Stickleback	—	—	11 (12)*	0	—	—

* Livers were bulked before fractionation. † Trace of activity was detected.

In regenerating liver, 4 days after partial hepatectomy, there was a definite ($P<0{\cdot}001$) increase in the activity of sulphatase $A+B$ but little change in any of the other sulphate enzymes. In necrotic liver, 36 hr. after the administration of CCl_4, there appeared to be a decrease in the activity of steroid sulphatase ($P<0{\cdot}02$) but there was no significant change in the levels of any of the other enzymes. Abbott & East (1949) claimed to have detected a fall in rat-liver arylsulphatase activity after administration of CCl_4 but it is unfortunately not possible from the few details published to decide which of the various arylsulphatases was in fact being studied by these authors.

The sex difference in the ability to synthesize DHAS would seem to lie in the presence of differing amounts of the appropriate sulphotransferase in the two sexes rather than in any difference in the ability to synthesize 'active sulphate' (Robbins & Lipmann, 1956), as there is no sex difference in the rate of synthesis of phenyl sulphate by similar liver preparations. The synthesis of phenyl sulphate was assayed under the conditions used for the assay of DHAS synthesis except that the substrate was mM-phenol. Four preparations from the livers of male rats synthesized phenyl sulphate at a mean rate of 147 ± 43 μm-moles/hr./g. wet wt., whereas the corresponding mean value for four preparations from female rats was 125 ± 15 μm-moles/hr./g. It is obvious that for these no sex difference exists. This is further evidence for the view (Gregory & Nose, 1957) that two independent sulphotransferases are involved in the synthesis of aryl and steroid sulphates.

Mouse. The animals were used at an age of $2\frac{1}{2}$–3 months. The general level of all the sulphate enzymes in mouse liver was very much lower than that in the rat (Table 3). There was no convincing sex difference in any of the sulphatases, the apparent greater sulphatase $A+B$ activity in the male not being highly significant ($P<0{\cdot}05$). With the DHAS-synthesizing system, however, there was a highly significant sex difference ($P<0{\cdot}001$) comparable with that in the rat.

In regenerating liver, 4 days after partial hepatectomy, there was a considerable increase in the activity of sulphatases $A+B$. In necrotic liver, 36 hr. after the administration of CCl_4, there was a similar rise in the activity of sulphatases $A+B$ ($P<0{\cdot}01$), together with a fall in the level of sulphatase C activity ($P<0{\cdot}001$). No steroid sulphatase activity whatever could be detected in necrotic mouse liver. In neither case was there any change in the ability to synthesize DHAS.

Guinea pig. In this species neither sulphatase C nor steroid sulphatase could be detected, either in the microsomes or in preparations obtained by the method used to prepare sulphatase C from ox liver (Roy, 1956*c*). With prolonged periods of incubation (up to 24 hr.) the hydrolysis of NPS by cell fractions could be detected, but the activity of the mitochondria was greater than that of the microsomes, and in both the activity was greater at pH 6 than at pH 8, suggesting that enzymes of the sulphatase A or B types were involved. This is in agreement with the work of Morimoto (1937) and of Tanaka (1938), who found the pH optimum for the hydrolysis of NPS by guinea-pig liver to be in the region of pH 6.

As seen from the results in Table 3, there was no significant sex difference in either the level of sulphatases $A+B$ or the DHAS-synthesizing system.

During the period of the experiments a *Salmonella* infection occurred in the guinea-pig colony; the sulphatase activities of the livers of these infected animals were very much higher than normal ($P<0{\cdot}01$). Unfortunately the causative organism was not identified, but it was almost certainly *Salmonella typhimurium*. The sulphatase activity of the livers of scorbutic guinea pigs also seemed to be rather higher than normal. In animals laying down connective tissue in response to carrageenin injection 4 days previously no significant differences in the levels of these enzymes could be detected.

Other species. Results obtained with the other species studied are summarized in Table 3. The main point to note is that sulphatase C could not be detected in the livers of the frog (*Rana temporaria*) and of the stickleback (*Gasterosteus aculeatus*). Steroid sulphatase was also lacking from the former species.

Occurrence of sulphatases A *and* B

It has already been shown that there are at least two soluble sulphatases, sulphatases A and B, in the livers of the ox and the rat (Roy, 1953*a*, 1954*a*), and it was of interest to study these in other species. From the shapes of the pH–activity curves, shown in Fig. 2, it seemed likely that enzymes corresponding to both sulphatases A and B were present in all the species investigated, with the possible exception of the frog, as those curves were analogous to those previously obtained (Roy, 1954*a*) in an investigation of ox liver, known to contain both sulphatases A and B. The pH curve of the sulphatase of stickleback liver was similar to that of the frog.

The results of experiments designed to separate sulphatases A and B by paper electrophoresis are summarized in Fig. 3. Enzymes corresponding to sulphatases A and B were detected in all the species studied except the guinea pig, the hen, the frog and the stickleback. In the frog the arylsulphatase seemed to be a pure B type of enzyme,

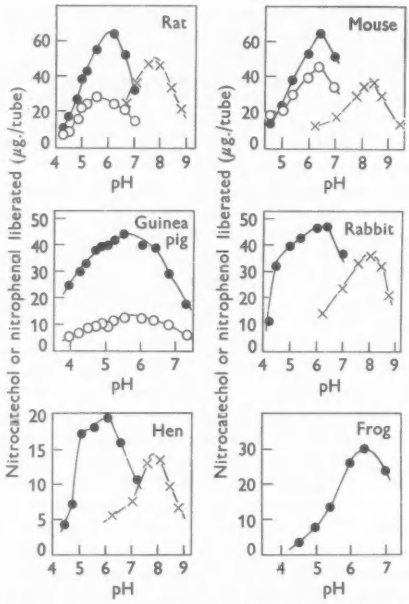

Fig. 2. Effect of pH on the rate of hydrolysis of NCS and NPS by cell fractions in various species. Conditions were as specified in Table 1, apart from variations in pH. Hydrolysis of NCS by mitochondria, ●; by microsomes, ○. Hydrolysis of NPS by microsomes, ×.

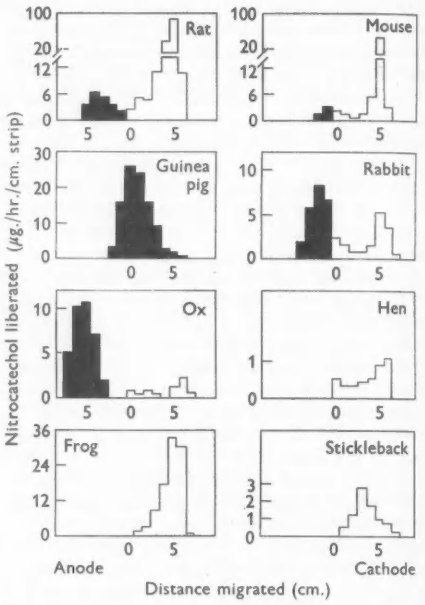

Fig. 3. Electrophoretic separation of the soluble sulphatases of various species. Enzyme preparations were obtained as described in text. Run for 16 hr. at pH 7·4 in 0·03 M-diethylbarbiturate buffer. Assays were carried out at pH 5 in 3 mM-NCS (black areas) and at pH 6 in 0·03 M-NCS (clear areas).

as shown by its behaviour on electrophoresis (Fig. 3), by its pH curve (Fig. 2) and by its substrate concentration–activity curve (Fig. 4), the latter giving a value for the K_m in the region of 0·01 M-NCS, a value comparable with that of a mammalian type *B* arylsulphatase. The situation in the stickleback was apparently identical with that in the frog. In the guinea pig the situation was quite different; although only one arylsulphatase could be detected by paper electrophoresis (Fig. 3), the shape of the pH curve (Fig. 2) suggested that more than one such enzyme was present. Attempts to prepare two enzymes by the methods used to obtain ox-liver sulphatases *A* and *B* (Roy, 1953*b*, 1954*a*) were not successful, but the results were consistent with more than one arylsulphatase being present. In the hen also the pH curve (Fig. 2) would suggest that both sulphatases *A* and *B* were present, although they could not be detected by electrophoresis (Fig. 3). However, the arylsulphatases of the hen were apparently much less stable during the electrophoresis than were those of the other species studied, as the total amount of activity recovered after electrophoresis was only a very small proportion of that originally

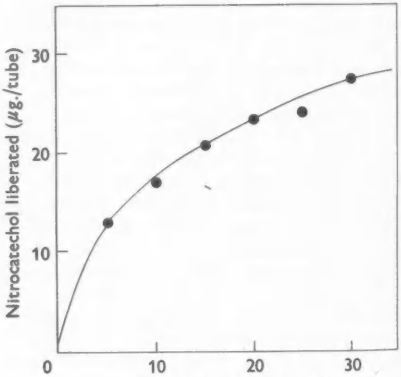

Fig. 4. Effect of variations in substrate concentration on the hydrolysis of NCS by frog-liver mitochondria at pH 6·4.

Table 4. *Intracellular distribution of the aryl sulphatases*

Assays were carried out as listed in Table 1. Numbers of animals were as shown in Table 3. Figures are percentage of total recovery found in each fraction. N, Nuclei; M, mitochondria; P, microsomes; S, supernatant.

		Sulphatases $A+B$				Sulphatase C			
Species	Sex	N	M	P	S	N	M	P	S
Rat	M	7±1	45±4	32±5	19±4	5±4	8±8	88±8	0
	F	8±1	44±6	29±3	19±4	0	0	100	0
Mouse	M	7±1	42±4	29±2	21±4	0	0	100	0
	F	13±9	46±8	21±5	20±15	0	0	100	0
	F*	10±2	31±1	27±1	32±2	0	0	100	0
Guinea pig	M	8±2	38±8	12±2	43±8	—	—	—	—
	F	11±3	45±5	13±3	33±5	—	—	—	—
Rabbit	M	15	34	16	36	0	0	100	0
	F	15	32	14	39	0	0	100	0
Ox	M	14	47	20	20	13	5	66	18
Hen	F	8	27	47	19	11	27	63	0
Frog	M	12	26	10	52	—	—	—	—
Stickleback	—	23	58	19	0	—	—	—	—

* Liver necrosis was induced by CCl_4.

present. This instability might well account for the apparent absence of a sulphatase *A* from hen liver.

The ratio of sulphatase *A* to sulphatase *B*, as determined by paper electrophoresis, varies considerably in the different species. The ratio of sulphatase *A* to sulphatase *B* activity ranges from 0·1 in the rat and the mouse through 2 in the rabbit to 6·5 in the ox. These values are only approximate, as the method cannot take into account the differing stability of the two enzymes to paper electrophoresis, nor the complicating effects of the anomalous kinetics of sulphatase *A*, which become important at the high dilutions obtained after electrophoretic separation of the enzymes.

Intracellular localization of the arylsulphatases

It has previously been claimed that, in both the mouse and the rat (Roy, 1953*a*, 1954*b*), the greater part of the sulphatase *A* and *B* activity is associated with the mitochondria. These claims are supported by the results shown in Table 4. The distributions found in the other species are in general agreement with the original observations, although in the guinea pig, and to an even greater extent in the frog, a high proportion of the sulphatase $A+B$ activity occurs in the soluble fraction. It might well be that this does not reflect a fundamental change in the intracellular distribution of these enzymes in those species, but rather that their sulphatase-containing granules are more readily disrupted, so liberating the enzymes in a soluble form during the isolation procedure.

The solubility of the sulphatase $A+B$ activity of the mitochondrial and microsomal fractions is shown in Table 5. In this connexion a soluble

Table 5. *Solubility of sulphatases* A *and* B *in mitochondria and microsomes*

A 'soluble' enzyme is defined as one not sedimentable in 1 hr. at 24 000 *g*. Values are expressed as percentage of whole fraction.

Fraction		Rat	Mouse
Mitochondria	Total	100	100
	Soluble (*a*)	79	75
	Insoluble (*b*)	35	32
	$a+b$	114	107
Microsomes	Total	100	100
	Soluble (*a*)	33	26
	Insoluble (*b*)	52	61
	$a+b$	85	87

enzyme is defined as being one which is not sedimented on centrifuging the suspension of the particles in water for 1 hr. at 24 000 *g* at 0°.

Results shown for sulphatase *C* in Table 4 confirm the observations of Dodgson, Spencer & Thomas (1954) and show this enzyme to have a purely microsomal origin. The amounts of sulphatase *C* occurring in fractions other than the microsomes are simply those to be expected from contamination of the various cell fractions by the former, with a possible exception for the hen, although the considerable amounts of lipid present in the liver of this species may have interfered with the fractionation. It should be noted that in those cases where sulphatase *C* was apparently restricted entirely to the microsomes some activity could be found in the other cell fractions by using longer periods of incubation than normal.

The various treatments listed in Table 3 had no significant effect on the intracellular distribution of the various sulphatases except for the administration

of CCl_4 to mice, which increased the proportion of the sulphatase $A+B$ activity found in the soluble fraction, with a corresponding decrease in the amount present in the mitochondria. This effect could well be due to the toxic action of the CCl_4 making the cell particles more readily lysed (Dianzani, 1957).

The significance of the cell fractions obtained from the livers of the lower vertebrates has not been investigated. These fractions may be only distantly related to the corresponding fractions of mammalian liver, but this is of little significance in the present connexion. A consideration of the results shown in Table 4, however, suggests that the cell fractions obtained from the livers of the lower vertebrates are directly comparable with those obtained from mammalian liver.

DISCUSSION

As shown by the results summarized in Table 2 the method would seem to be a useful one for the determination of sulphatases in liver tissue, the only serious disadvantage being the tedious nature of the procedure. It would seem also that the method could be adapted without difficulty to any other tissue from which the appropriate cell fractions can be prepared. At present there is no way of assessing the absolute significance of the results, at least with regard to sulphatases *A* and *B*, as it is impossible to determine these enzymes in unfractionated preparations. With sulphatase *C*, however, the results for the rat are of the same order as those obtained by Dodgson, Spencer & Thomas (1953) with potassium *p*-acetylphenyl sulphate as substrate with unfractionated-liver preparations.

The results of the comparative studies need little comment. Sulphatases of the type of ox-liver sulphatase *B* (Roy, 1954*a*) have been found in all the vertebrate species studied, and these may be accompanied in the higher species by sulphatases comparable with ox-liver sulphatases *A* and *C* (Roy, 1953*b*, 1956*c*). Only in the guinea pig does a sulphatase comparable, at least in some respects, with sulphatase *A* occur without the simultaneous occurrence of a sulphatase *C*. Although sulphatases of the *A* and *B* type seem to be of ubiquitous occurrence, at least in the higher vertebrates, the results summarized in Fig. 3 show that there can be very considerable variations in the proportions of these two enzymes. The two extremes seem to be the rat and the ox; in the former a sulphatase of the *B* type accounts for by far the greater part of the sulphatase $A+B$ activity, whereas in the latter a sulphatase *A* is the predominant enzyme. In the guinea pig the sulphatases hydrolysing NCS, and presumably of the *A* or *B* types, appear to differ from the corresponding enzymes in all the other species studied; this is reflected not only by their behaviour on electrophoresis (Fig. 3) but also by the position of their optimum substrate concentration (Table 1).

Those species which are lacking in sulphatase *C* also seem to be deficient in steroid sulphatase. As shown by the results in Table 3 there seems to be a very close relationship indeed between these two enzymes. It has already been suggested (Roy, 1957*a*) on indirect evidence that these two activities are due to separate enzymes, but until direct evidence is obtained the possibility that only one enzyme is responsible for both types of activity cannot be ignored.

It is interesting to compare the situation in the vertebrates with that in the few invertebrate species so far studied. Electrophoretic (Roy, 1956*a*) and kinetic (Dodgson, Lewis & Spencer, 1954; Roy, 1956*a*) studies have shown that the arylsulphatase of the limpet, *Patella vulgata*, is comparable in many respects with a mammalian sulphatase of the *A* type. Similarly in the snail, *Helix pomatia*, the arylsulphatase would seem to be of the *A* type, to judge by its kinetic properties and its behaviour on electrophoresis (Jarrige & Henry, 1952). Again, in the locusts *Locusta migratoria* and *Schistocerca gregaria* the arylsulphatase seemed to be of an *A* type (Robinson, Smith & Williams, 1954). Dodgson, Lewis & Spencer (1954) did not detect in the limpet any sulphatase of the *C* type under conditions in which its presence should have been obvious had it occurred in significant amounts. In the few invertebrates so far studied therefore the sole arylsulphatase present would seem to be of the type of mammalian sulphatase *A*, in rather striking contrast with the situation in the lower vertebrates where the arylsulphatase is of the *B* type. It should be noted, however, that the invertebrate sulphatases do not show the kinetic anomalies of mammalian sulphatases of the *A* type. Another striking difference in the invertebrate sulphatases is seen in the steroid sulphatase; in mammals this enzyme is insoluble and has a pH optimum in the region of 8; in the mollusc it has a pH optimum of approximately 5 and is readily soluble (Roy, 1956*a*).

The changes in the levels of the sulphate enzymes under various experimental conditions are listed in Table 3 and little of a general nature can be said. The different behaviour of the sulphatases in the livers of rats and mice poisoned with carbon tetrachloride may well be due to the differing responses of these species to that substance: histological examination of the livers of animals treated with carbon tetrachloride showed that the amount of tissue damage was considerably less in the rat than in the mouse. This could well be the reason for the

differing responses in the two species. It seems likely that the rise in the sulphatase activity in the livers of scorbutic guinea pigs was due to the general changes induced in liver by ascorbic acid deficiency (Christian, 1947) rather than to any specific effect on the sulphatases. The only general conclusion which it seems possible to draw from this result is that sulphatases *A* and *B* (either or both) are present in increased amounts under conditions in which cell proliferation is occurring. There is also apparently a tendency for sulphatase *C*, and the associated steroid sulphatase, to be low in necrotic liver.

It is interesting to note the almost exact parallelism in the responses of sulphatases *A*+*B* and of β-glucuronidase (Levvy *et al.* 1948) in mouse liver to partial hepatectomy and to the administration of carbon tetrachloride. In the rat the parallelism was not so exact, but in this species the behaviour of β-glucuronidase is complicated by the presence of an endogenous inhibitor and also by the important strain differences between Wistar and Lister rats (Walker & Levvy, 1953). Such a close relationship between β-glucuronidase and sulphatases *A* and *B* is perhaps not unexpected if, as is discussed below, both enzymes occur in the lysosomes.

The results confirm the earlier claims (Dodgson, Spencer & Thomas, 1954; Gianetto & Viala, 1955) that sulphatase *C* is of purely microsomal origin and are also in agreement with the previous observations (Roy, 1953*a*, 1954*b*) that in the mouse and the rat the highest proportion of sulphatases *A* and *B* is localized in the mitochondrial fraction, although considerable amounts may be found in the other cell components. These results are apparently consistent with the claim that sulphatases *A* and *B* occur in the lysosomes (Viala & Gianetto, 1955), as these particles distribute themselves between the mitochondrial and microsomal fractions obtained by the usual procedure (Applemans, Wattiaux & de Duve, 1955). The present results are not in complete agreement with this view, however, as according to Viala & Gianetto (1955) when lysosomes are suspended in water all the associated sulphatase activity is liberated in a soluble form. As seen from Table 5 this is not the case. A considerable proportion of these sulphatases remains insoluble when the mitochondria, and more especially the microsomes, are treated with water. This is obviously not in accord with the views of Viala & Gianetto (1955) and it suggests that the sulphatases *A* and *B* of these two cell fractions are 'bound' in different ways. Again there is the observation (Roy, 1956*c*) that in the ox some of the sulphatases of this type are very closely associated with sulphatase *C* and remain insoluble, despite prolonged treatment with water: this would again suggest that there may be more than one form of sulphatases *A* and *B* in liver. The detailed description of the intracellular distribution of these enzymes is therefore still in doubt although the general picture is certainly that summarized in Table 4. It would seem reasonable to conclude that in all probability the view that sulphatases *A* and *B* are associated with the lysosomes is partly correct, but the possibility that some of these enzymes are associated with the true mitochondria, or more likely with the true microsomes, cannot be excluded.

Perhaps the most important general conclusion to be drawn from the work is that, apart from the possible interdependence of sulphatase *C* and steroid sulphatase, each of these enzymes can vary quite independently of one another, suggesting that each of them has a specific role to play in the metabolic processes of the animal. No conclusion about the function of the arylsulphatases can be drawn and consideration of the fact that one or more of these enzymes may be lacking from some species suggests that they cannot be fulfilling any very important role in the normal animal. The function of sulphatases *A* and *B* must be considered in relation to the function of the many other hydrolases which occur in the lysosomes; de Duve, Pressman, Gianetto, Wattiaux & Appelmans (1955) have suggested that they play some role in localized intracellular digestion, but there is no evidence for, or against, this hypothesis.

SUMMARY

1. To minimize the difficulties inherent in the assay of the arylsulphatases in unfractionated tissue preparations these enzymes were determined in liver-cell fractions obtained from a sucrose homogenate of the tissue. Sulphatases *A* and *B* were together determined with nitrocatechol sulphate as substrate and sulphatase *C* was assayed with *p*-nitrophenyl sulphate as substrate.

2. Values are given for the arylsulphatase activities of the livers of several species. Steroid sulphatase and the steroid sulphate-synthesizing system were also assayed and the values are reported.

3. Sulphatase *C* and steroid sulphatase activities were much higher in the livers of male than of female rats. The level of the steroid sulphate-synthesizing system was considerably higher in the livers of female rats and mice than in the corresponding male animals.

4. The changes in these activities caused by various experimental procedures are described. The level of sulphatases *A* and *B* appeared to be elevated in proliferating tissues.

5. The approximate relative proportions of

sulphatases *A* and *B* were determined by paper electrophoresis. There were considerable variations in this ratio and sulphatase *A* appeared to be lacking in the lower vertebrates.

6. Sulphatases *A* and *B* were localized predominantly in the mitochondrial and microsomal fractions. Sulphatase *C* occurred only in the microsomes.

7. Although the distribution of sulphatases *A* and *B* was consistent with them occurring in the lysosomes they could not be completely extracted from the mitochondrial and microsomal fractions with water, so that it is probable that not all of these enzymes are localized in the lysosomes.

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The Biosynthesis of Penicillin

8. INVESTIGATION OF CYCLIC CYSTEINYLVALINE PEPTIDES AS PRECURSORS*

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The demonstration of the intact incorporation of L-cystine, presumably after reduction to cysteine (Arnstein & Grant, 1954*a*, *b*; Arnstein & Crawhall, 1957) and of the utilization of the carbon chain of valine for the biosynthesis of the β-lactam–thiazolidine ring system of penicillin (I) (Arnstein & Grant, 1954*a*; Stevens, Vohra & De Long, 1954; Arnstein & Clubb, 1957) has led to speculation on the mechanism by which these amino acids combine, and the nature of the more immediate precursors of the penicillin molecule so formed.

Although the utilization of valine nitrogen by

* Part 7. Arnstein & Margreiter (1958).

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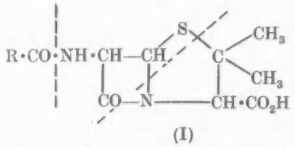
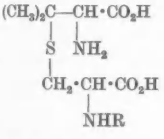
the mould could not be demonstrated conclusively owing to the rapid transamination which it undergoes on addition to the fermentation medium (Arnstein & Clubb, 1957), D-valine has now been excluded as a penicillin precursor (Arnstein & Margreiter, 1958). It is probable therefore that the penicillamine moiety of penicillin is derived from either L-valine or the corresponding keto acid, α-oxo*iso*valeric acid. Several metabolic pathways by which these compounds might combine with cysteine may be postulated, and of these some of the more obvious have already been excluded. For example, the formation of ββ-dimethyl-lanthionine (II) in a manner analogous to that of cystathionine in *Neurospora* (Horowitz, 1947), that is by condensation of cystine with β-hydroxyvaline, does not appear to take place (Arnstein & Clubb, 1957; Stevens, Vohra, Moore & De Long, 1954).



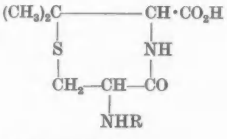
(I)

(R=$C_6H_5 \cdot CH_2$ in benzylpenicillin)

Phenylacetylcysteine, from which phenylacetyldimethyl-lanthonine (VIII) could be formed without involving dimethyl-lanthionine (II) itself, has also been excluded (Arnstein, Clubb & Grant, 1954). It therefore seemed likely that a peptide bond is first formed between cystine (or cysteine) and valine and that the thio ether linkage is introduced subsequently by the intramolecular cyclization of an intermediate such as cysteinyl-β-hydroxyvaline or cysteinyl-αβ-dehydrovaline. A reaction of this kind would give rise to a cyclic derivative (VI) of cysteinylvaline containing both thio ether and peptide bonds in a seven-membered ring.



(II; R = H)
VII, R = CO·CH_3
VIII, R = CO·$CH_2 \cdot C_6H_5$



(III; R = CO·CH_3)
IV, R = CO·$CH_2 \cdot C_6H_5$
V, R = CO·O·$CH_2 \cdot C_6H_5$
VI, R = H

The present paper describes the synthesis of peptides of this type, and preliminary experiments designed to test whether they are involved as intermediates in penicillin biosynthesis. Since the synthesis of these compounds was lengthy and the overall yields were low, an isotopic-competition method was used, ^{14}C-labelled valine being added alone or together with the peptides to a suspension of mycelial cells synthesizing the antibiotic. A reduction in the specific radioactivity of penicillin synthesized in the presence of the unlabelled peptides would indicate that they are more immediate precursors than valine itself. Contrary to expectation, no indication of the utilization of these compounds for penicillin biosynthesis was obtained and further investigations with labelled material were therefore not undertaken. Two substituted thio ether compounds related to ββ-dimethyl-lanthionine, namely *S*-(2-acetamido-2-carboxyethyl)-DL-penicillamine (VII) and *S*-(2-phenylacetamido-2-carboxyethyl)-DL-penicillamine (VIII), resulting from the acid hydrolysis of (III) and (IV) respectively, were also included in the tests but proved equally ineffective in reducing the incorporation of labelled valine into penicillin.

EXPERIMENTAL

Radioactivity measurements. Compounds were mounted on 1 sq.cm. polythene disks and counted as samples of 'infinite thickness', by using a thin end-window Geiger–Müller tube (Popják, 1950). All counts were compared with a poly[^{14}C]methyl methacrylate reference standard (1 μc/g.) obtained from the Radiochemical Centre, Amersham. The standard error in all determinations is less than 5%.

Method for incubation of washed mycelial cells. Washed mycelium of *Penicillium chrysogenum* WIS 51–20, prepared as described by Halliday & Arnstein (1956), was used at a concentration of 0·1 g. wet wt./ml. of medium, the dry wt. being 10–15% of the wet wt. The incubation medium contained 0·005% of potassium phenylacetate, 0·25% of glucose, and 0·005% of L-[*carboxy*-^{14}C]valine (0·0289 μc/mg.) and the suspension of mycelium was incubated in duplicate conical flasks (250 ml. for 40 ml. of medium) on a rotary shaker (250 rev./min.) at 24° for 4 hr. The compounds to be tested were added at a final concentration of 0·025%. The penicillin concentration of the medium was determined by cup-plate bioassay with *Bacillus subtilis* (I.C.I. strain NCTC 8241) as test organism (Humphrey & Lightbown, 1952) at the end of the experiment, and the rate of synthesis under these conditions was about 4 i.u./ml./hr.

Isolation and purification of penicillin. Inactive carrier penicillin (40 mg. of Glaxo sodium benzylpenicillin, potency 1650 i.u./mg.) was added to the incubation broth after removal of mycelium by filtration and sampling for bioassay. The solution was cooled to 4° and acidified with 25% (v/v) phosphoric acid to pH 2, and the penicillin extracted with ether. The volume of the solution was reduced by successive partitioning of penicillin between ether at pH 2 and phosphate buffer at pH 7·0 (Smith & Hockenhull, 1952). Finally, the penicillin was isolated as the *N*-ethylpiperidine salt (28–30 mg.) which was purified to constant radioactivity by recrystallization from chloroform–acetone (1:4, v/v).

Chemical preparations

L-[carboxy-^{14}C]*Valine.* This material was obtained by resolution of DL-[*carboxy*-^{14}C]valine supplied by the Radio-

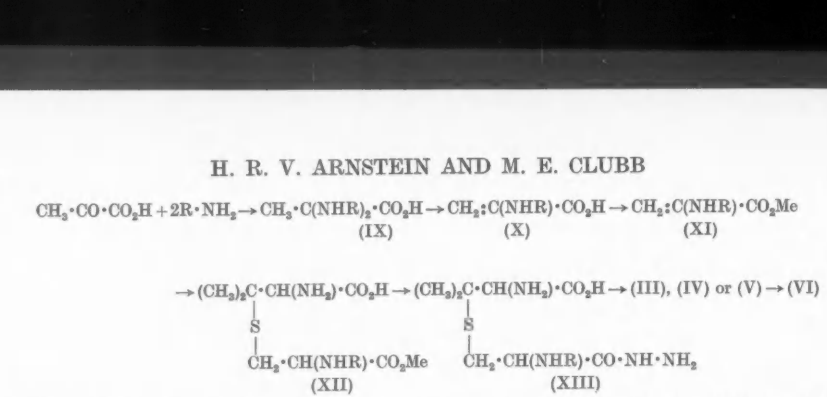

CH2·CO·CO2H + 2R·NH2 → CH3·C(NHR)2·CO2H → CH2:C(NHR)·CO2H → CH2:C(NHR)·CO2Me
(IX) (X) (XI)
→ (CH2)2C·CH(NH2)·CO2H → (CH2)2C·CH(NH2)·CO2H → (III), (IV) or (V) → (VI)
S S
CH2·CH(NHR)·CO2Me CH2·CH(NHR)·CO·NH·NH2
(XII) (XIII)

Fig. 1. Synthesis of cyclic peptides related to penicillin. (R = CH_3·CO, C_6H_5·CH_2·CO or C_6H_5·CH_2·O·CO).

Table 1. *R_F values of peptides and intermediates*

All paper chromatograms were run on Whatman no. 1 paper. Solvent systems had the following composition (by vol.): *A*, Butanol–acetic acid–water (63:10:27); *B*, pentan-1-ol–pyridine–water (7:7:6); *C*, phenol–water (5:2) (NH_3 present in the chromatography tank).

All substances were detected by spraying the paper with 0·1% ninhydrin in butanol containing 2% of water, unless otherwise stated. For chemical formulae of the substances see text and Fig. 1. – Signifies value not determined.

Substance	Solvent systems *A*	*B*	*C*
(XII; R = CH_3·CO)	0·38	0·32	–
(XIII; R = CH_3·CO)	–	0·48	0·85
(III)	0·69*	0·25*	–
(XII; R = C_6H_5·CH_2·CO)	0·66	0·35	–
(XIII; R = C_6H_5·CH_2·CO)	0·61	0·53	–
(IV)	0·78–0·84†	–	–
(XII; R = C_6H_5·CH_2·O·CO)	0·63–0·66	0·38	–
(XIII; R = C_6H_5·CH_2·O·CO)	0·69	–	–
(V)	0·82–0·88*†	–	–
(VI)	0·28–0·33	0·25	0·78
(VII)	0·16	0·23	0·53
(VIII)	0·33–0·43	0·06	0·66

* Material detected by chlorine–starch–iodide technique (Rydon & Smith, 1952) and also with bromophenol blue and methyl red indicators. Repeated attempts to detect the cyclic phenylacetylpeptide (IV) by these methods were, however, unsuccessful.

† Material detected by spraying with 1% $AgNO_3$ in aq. N-NH_3, drying and heating the paper at 115°, when light spots on a brown background were obtained. Alternatively BDH Universal Indicator (British Drug Houses Ltd.) could be used, but the spots were more difficult to detect.

chemical Centre, Amersham, by means of the brucine salts of *N*-formyl-DL-valine (Arnstein & Clubb, 1957).

Synthesis of peptides. The method used for synthesizing the cyclic peptides (III)–(VI) is outlined in Fig. 1. All melting points were measured on a micro-block apparatus and are uncorrected. The R_F values of peptides and intermediates are given in Table 1.

6-*Acetamido*-2:2-*dimethyl*-5-*oxohexahydro*-1:4-*thiazepine*-3-*carboxylic acid* (*III*). Freshly distilled pyruvic acid (44 g., 0·5 mole) was condensed with acetamide (59 g., 1·0 mole, twice recrystallized from acetone) by azeotropic distillation in toluene (150 ml.) at a bath temperature of 160°. After 3 hr. 90% of the theoretical volume of water had been collected. The toluene was decanted and the brown crystalline residue washed with warm ethanol, yielding diacetamidopropionic acid (IX; R = CH_3·CO) (46·5 g., 50%), m.p. 197°.

The acid (IX; 15 g.) was partially hydrolysed in acetic acid containing 1 drop of conc. HCl/100 ml. (cf. Bergmann & Grafe, 1930). Approximately half the acetic acid was removed by evaporation *in vacuo*, and on cooling acetamidoacrylic acid (X; R = CH_3·CO) crystallized in fine needles. The product was washed with ice-cold water (3 × 10 ml.) and dried, m.p. 205° (yield 7·8 g., 75%).

Acetamidoacrylic acid was esterified according to Rothstein (1949) and the methyl ester (XI; R = CH_3·CO) was obtained as almost colourless needles (yield 73%), m.p. 52–53° after crystallization from light petroleum (b.p. 40–60°).

The product (6 g., 42 m-moles) was dissolved in water (137 ml.) and added to a solution in water (80 ml.) of *iso*propylidene-DL-penicillamine hydrochloride (4·7 g., 42 m-moles) which had been boiled for 3 min. to hydrolyse the thiazolidine and neutralized with aq. 1·5N-NH_3. The mixed solution was heated in a steam bath with a slow stream of H_2 passing through. After 3 hr. the solution gave a negative thiol reaction with alkaline sodium nitroprusside (cf. Süs, 1950). Inorganic salt was removed from the solution by the addition of 1% silver acetate (700 ml.). Hydrogen sulphide was passed through and AgCl and Ag_2S were removed simultaneously by centrifuging. The supernatant solution was evaporated to dryness and the brown residue was extracted continuously with hot ethanol. S-(2-*Acetamido*-2-*methoxycarbonylethyl*)*penicillamine* (XII; R = CH_3·CO) was obtained as a white microcrystalline solid on addition of an equal volume of ether to the ethanol extract (5·8 g., m.p. 175–176°; second crop 4·8 g., m.p. 166°; total yield 85%). A sample was recrystallized from water–ethanol, m.p. 175–176° (Found: C, 45·1; H, 7·1; N, 9·5; S, 11·0. $C_{11}H_{20}O_5N_2S$ requires C, 45·2; H, 6·8; N, 9·6; S, 11·0%).

The ester (XII; 5·8 g., 20 m-moles) was suspended in cold ethanol (250 ml.) and 100% hydrazine hydrate (3·75 ml., 75 m-moles) was added. On warming, a clear solution was obtained and was kept at 37° for 60 hr. The ethanol was removed by evaporation *in vacuo* at a temperature not exceeding 50°. S-(2-*Acetamido*-2-*hydrazinocarbonylethyl*)*penicillamine* (XIII; R = CH_3·CO) was obtained as a white amorphous solid (2·3 g., 40%) by dissolving the residue in a small volume of water and adding ethanol to turbidity. The product was recrystallized from water–ethanol, m.p. 205–219° with decomposition (Found: C, 39·6; H, 7·0; N, 18·9; S, 10·6. $C_{10}H_{20}O_4N_4S$ requires C, 41·1; H, 6·9; N, 19·2; S, 11·0%).

Cyclization of the hydrazide (XIII; R = CH_3·CO) was effected at high dilution in aqueous medium, pH 7·0 (cf. Sheehan & Richardson, 1954). The above product (2·57 g., 8·75 m-moles) was dissolved in 0·2N-HCl (87·5 ml., 17·5 m-moles) and cooled to 0°. Sodium nitrite (0·605 g., 8·75 m-moles) in water (4·4 ml.) was added and the mixture swirled gently at 0° for 15 min. It was then poured into ice-cold water (5 l.), $NaHCO_3$ (11 g.) was added, and the solution was stored at 4° for 40 hr. The pH was adjusted to 4·5 by the addition of 2N-HCl (approx. 60 ml.) and the solution was concentrated to 135 ml. *in vacuo* at <45°. This solution was extracted with butanol (3 × 50 ml.), the extract concentrated *in vacuo* (to 100 ml.) and inorganic salt, which separated as a fine white cryst. solid on storage at 4° overnight, was removed. The butanolic solution was evaporated to dryness and the residue was crystallized from acetone (m.p. 215°; yield 0·9 g., 40%). On slow crystallization from water, the *cyclic peptide* (III) was obtained as fine needles, m.p. 215–216° [Found: C, 45·9; H, 6·2; N, 10·6; S, 12·7%; mol.wt. (Rast) 310. $C_{10}H_{16}O_4N_2S$ requires C, 46·1; H, 6·2; N, 10·8; S, 12·3%; mol.wt. 260]. This material gave no colour with ninhydrin but was detected on paper chromatograms by the chlorine–starch–iodine technique (Rydon & Smith, 1952) and with suitable indicators (see Table 1).

2:2-Dimethyl-5-oxo-6-phenylacetamidohexahydro-1:4-thiazepine-3-carboxylic acid (*IV*). This compound was prepared by a series of reactions analogous to those for the preparation of (III). Phenylacetamidoacrylic acid, m.p. 180–183°, was prepared by the method of Süs (1950). The *methyl ester* (XI; R = C_6H_5·CH_2·CO) was obtained by refluxing the silver salt with methyl iodide for 3 hr. On crystallization from light petroleum (b.p. 60–80°) it formed white plates, m.p. 56° (yield 50%) (Found: C, 65·9; H, 5·9; N, 6·2; $C_{12}H_{13}O_3N$ requires C, 65·8; H, 6·0; N, 6·4%).

The ester (6·0 g., 29·5 m-moles) was dissolved in ethanol (100 ml.) and heated on a steam bath in a slow stream of H_2 for 3 hr. with a solution of *iso*propylidenepenicillamine hydrochloride (6·8 g., 30 m moles) in water (100 ml.) which had been boiled for 3 min., cooled and neutralized with 1·5N-NH_3. The reaction mixture was freed from inorganic salt and purified as before. The *product* (XII, R = C_6H_5·CH_2·CO) (5·9 g., 54%) was obtained as a white powder from ethanol–ether, m.p. 164° (Found: C, 54·6; H, 6·7; N, 7·5; S, 8·9. $C_{17}H_{24}O_5N_2S$ requires C, 55·4; H, 6·6; N, 7·6; S, 8·7%).

The ester (XII; R = C_6H_5·CH_2·CO) (5·6 g., 15 m-moles) was suspended in ethanol (400 ml.), and 100% hydrazine hydrate (3·0 ml.) was added. The mixture was kept at 37° for 16 hr. and then heated on a steam bath for a further 6 hr. Removal of the ethanol by evaporation *in vacuo* and crystallization of the residue from water–ethanol gave the *hydrazide* (XIII; R = C_6H_5·CH_2·CO) as a white crystalline powder (1·35 g., 24%), m.p. 219° with decomposition (Found: C, 52·0; H, 6·1; N, 15·1; S, 9·0. $C_{16}H_{24}O_4N_4S$ requires C, 52·2; H, 6·6; N, 15·2; S, 8·7%).

The hydrazide (0·81 g., 2·2 m-moles) was converted into the acid azide and cyclized at high dilution (1·5 l.) as described above for the acetyl compound. After purification as for (III), the *cyclic peptide* (IV) crystallized from a small volume of water in fine needles (0·2 g., 27%), m.p. 194–195° (Found: C, 56·9; H, 6·1; N, 8·5; S, 9·7%; mol.wt. (Rast) 347. $C_{16}H_{20}O_4N_2S$ requires C, 57·1; H, 6·0; N, 8·3; S, 9·5%; mol.wt. 336).

6-Amino-2:2-dimethyl-5-oxohexahydro-1:4-thiazepine-3-carboxylic acid (*VI*). Benzyl carbamate (50 g., 0·33 mole) was condensed with pyruvic acid (25 ml., 0·37 mole) by azeotropic distillation in benzene (250 ml.) for 2·5 hr. at a bath temp. of 110–115°. The residue, after the benzene had been decanted, crystallized on cooling and was recrystallized from ethyl acetate (yield, 15 g., 30%; m.p. 138–140°). Benzyl carbamate (approx. 20 g.) was recovered from the mother liquors. αα-Dibenzyloxycarbonamidopropionic acid (IX, R = C_6H_5·CH_2·O·CO) (15 g., 41 m-moles) was partially hydrolysed by the method of Frankel & Riechmann (1952) to α-benzyloxycarbonamidoacrylic acid, which was recrystallized from ethanol–water (yield 8 g., 85%; m.p. 103–105°).

The product (8 g., 36 m-moles) was converted into the silver salt (yield, 98%) which was stirred with methyl iodide (70 ml., 150% excess) and a trace of quinol at room temp. for 2 hr. The mixture was filtered and the solid refluxed with a further portion of methyl iodide (30 ml.) for 1 hr. Methyl iodide was removed from the combined filtrates at atmospheric pressure. The *methyl ester* (XI, R = C_6H_5·CH_2·O·CO) crystallized as dense needles when the residue was dissolved in light petroleum (b.p. 40–60°) and the solution cooled to −70°. It was recrystallized from the same solvent (yield 6·0 g.; 70%; m.p. 34–35°) (Found: C, 61·4; H, 5·7; N, 6·0. $C_{12}H_{13}O_4N$ requires C, 61·3; H, 5·6; N, 6·0%).

The methyl ester (5·88 g.; 25 m-moles) was condensed with penicillamine (25 m-moles) in ethanol–water (100 ml.: 50 ml.) containing a trace of quinol. The mixture was saturated with hydrogen and kept at 37° for 112 hr. After removal of inorganic salts as before, the *product* (XII, R = C_6H_5·CH_2·O·CO) was twice recrystallized from methanol–ether (yield 3·8 g., 40%; m.p. 172°) (Found: C, 53·1; H, 6·3; N, 7·0; S, 8·6. $C_{17}H_{24}O_6N_2S$ requires C, 53·1; H, 6·3; N, 7·3; S, 8·3%).

A solution of this ester (2·7 g., 6·75 m-moles) in methanol (75 ml.) containing 100% hydrazine hydrate (1·35 ml.) was kept at 37° for 72 hr. The solution was decanted off some crystals which had formed on the flask wall (0·65 g.; m.p. 225° with decomposition) and refluxed gently for 20 min., when more white solid formed (0·31 g.; m.p. 228°). A third crop of the hydrazide (0·17 g.; m.p. 225°) was obtained when more hydrazine hydrate (0·5 ml.) was added to the mother liquors and refluxed for a further 30 min. The *hydrazide* (XIII, R = C_6H_5·CH_2·O·CO) (total yield 1·13 g., 44%) was used without further purification but a sample was recrystallized from water–methanol (m.p. 225°) (Found: C, 48·8; H, 6·1; N, 14·5; S, 8·4. $C_{16}H_{24}O_5N_4S$ requires C, 50·0; H, 6·3; N, 14·6; S, 8·3%).

The hydrazide (0·96 g.; 2·5 m-moles) was converted into the acid azide and cyclized at high dilution (1·5 l.) as before. After being stored for 40 hr. at 4°, the solution was concentrated to 140 ml. *in vacuo*, cooled to 5°, and acidified to pH 4·5 with conc. HCl. After extraction with butanol (1 × 50, 2 × 25 ml.), the aqueous solution was neutralized to pH 7·0 and concentrated to 65 ml. A second butanol extraction (2 × 25 ml.) was made at pH 4·5, the combined extracts were dried over anhydrous Na_2SO_4 and lyophilized. The residue (0·7 g.) was thoroughly extracted with warm water (3 × 10 ml.) and the water-insoluble residue (0·6 g.) was crystallized from 50% aqueous acetone, ethanol or ethanol–water, yielding 0·34 g. (43%) of the *cyclic peptide* (V), m.p. 204° [Found: C, 54·4; H, 5·7;

N, 8·3; S, 8·7%; mol.wt. (Rast) 304; $C_{16}H_{20}O_5N_2S$ requires C, 54·5; H, 5·7; N, 8·0; S, 9·1%; mol.wt. 352].

The benzyloxycarbonyl group was removed by treating 100 mg. of the peptide (V) with M-HBr in acetic acid (5·0 ml.) (Ben-Ishai & Berger, 1952) for 2 hr. at room temp. in a flask fitted with a $CaCl_2$ tube. The mixture was warmed in a bath at 45–50° for a few minutes and lyophilized. The hydrobromide (68–72 mg., 80–85%) was washed with several portions of dry ether. The free base was obtained by dissolving the hydrobromide (50 mg.) in a little water, making alkaline with aq. NH_3 (sp.gr. 0·88) and evaporating the solution to dryness. The white crystalline powder was recrystallized from dil. aq. NH_3–ethanol (yield, 22·4 mg., 61%; m.p. above 250° with charring). The *product* (VI) gave a characteristic yellow colour reaction with ninhydrin, changing to mauve, and was chromatographically pure (see Table 1) (Found: C, 43·7; H, 6·6; N, 12·8; S, 15·8. $C_8H_{14}O_3N_2S$ requires C, 44·1; H, 6·5; N, 12·8; S, 14·7%).

S-(2-*Acetamido*-2-*carboxyethyl*)*penicillamine* (*VII*). Acid hydrolysis of (III) (200 mg.) with 12N-HCl (10 ml.) at 37° for 4 hr. resulted in the formation of (VII), as shown by paper chromatography. The mixture was frozen, HCl was removed *in vacuo*, and the free base obtained by passing an aqueous solution of the product through a column (diameter 1·5 cm.) containing Zeo-Karb 215 (10 g. wet wt. in H^+ form) and eluting with 0·033N-NH_3. By fractionation of the eluate (3·0 ml. fractions at the rate of 30/hr.) contaminating dimethyl-lanthionine (II) was removed. The *product* (VII) was crystallized from water–acetone and recrystallized from water–ethanol (yield 50·7 mg., 31%; m.p. 228°) (Found: C, 43·4; H, 6·4; N, 9·9; S, 10·9. $C_{10}H_{18}O_5N_2S$ requires C, 43·2; H, 6·5; N, 10·1; S, 11·5%). For comparison, samples (m.p. 220°) of (VII) were also prepared by saponification of the ester (XII, R=CH_3·CO) and by condensation of acetamidoacrylic acid with penicillamine (cf. Süs, 1950) (Found: C, 43·1; H, 6·8; N, 9·8; S, 11·5%). All these samples had identical R_F values in three different solvents (Table 1).

S-(2-*Phenylacetamido*-2-*carboxyethyl*)*penicillamine* (*VIII*). A sample of (VIII) was prepared by condensation of phenylacetamidoacrylic acid with penicillamine and was recrystallized from water (m.p. 196–197°; cf. Süs, 1950) (Found: C, 54·0; H, 6·0; N, 7·8; S, 8·9. $C_{16}H_{22}O_5N_2S$ requires C, 54·2; H, 6·3; N, 7·9; S, 9·0%). This material was identified by paper chromatography (see Table 1) with the product of hydrolysis of (IV) with 12N-HCl at 37° for 4 hr.

ββ-Dimethyl-lanthionine (*II*). *S*-(2-Acetamido-2-carboxyethyl)penicillamine (VII, 1·46 g., 5 m-moles) was boiled with 20% HCl (50 ml.) for 5 hr. The solution was evaporated to dryness, and passed through a column of Zeo-Karb 215 resin (H^+ form). After the column had been washed free from acid, the product was eluted with ammonia and crystallized from water–ethanol (yield 0·7 g., 59%; m.p. 250° with decomposition).

RESULTS

The yield and radioactivity of penicillin, synthesized by washed mycelium of *P. chrysogenum* in the presence of L-[*carboxy*-^{14}C]valine alone or together with the synthetic compounds whose precursor activity was to be tested, are shown in Table 2. The average production of penicillin was

Table 2. *Penicillin biosynthesis from* L-[carboxy-^{14}C]*valine in the presence of certain peptides and thio ethers related to cystine and valine.*

The compounds tested were added at a concentration of 25 mg./100 ml. to a suspension of washed mycelium in the presence of L-[*carboxy*-^{14}C]valine as described in the text. Penicillin production in control flasks was 12·5 i.u./ml. in Expts. nos. 1–4 and 10·5 i.u./ml. in Expts. nos. 5–7, the specific radioactivity of the penicillin *N*-ethylpiperidine salt being 2·54 and 2·23 μc/g. in the two series of experiments respectively.

Expt. no.	Compound tested	Yield of penicillin (i.u./ml.)	Specific radioactivity of penicillin *N*-ethylpiperidine salt (μc/g.)	Mean specific radioactivity (as % of control)
1	Acetyl peptide (III)	11·5 12·7	2·52 2·74	103·5
2	Phenylacetyl peptide (IV)	11·9 12·1	2·78 2·74	108·7
3	Dimethyl-lanthionine	11·8 12·3	2·89 2·96	114·9
4	Acetyldimethyl-lanthionine (VII)	13·6 12·1	2·36 2·74	100·4
5	Phenylacetyldimethyl-lanthionine (VIII)	11·2 6·5*	1·90 2·95	85·2
6	Amino peptide (VI)†	8·8 9·0	2·31 2·49	107·6
7	Phenylacetyl-L-cysteinyl-D-valine‡	10·1 5·6*	2·50 3·46	112·1

* Low penicillin yield: flask rejected in calculation of column 5.
† Compound added as hydrobromide.
‡ Supplied by Merck and Co. and added at a final concentration of 0·005% only.

not affected by the addition of the test compounds, being 98 and 93% (Expts. nos. 1–4 and 5–7 respectively) of the value in the corresponding control experiments. In Expt. no. 6 the hydrobromide of the peptide (VI) was used, but an equivalent amount of potassium hydroxide was added to maintain the neutral pH of the medium. In a further control experiment containing the same concentration of potassium bromide, the penicillin yield was shown to be unaffected by the salt.

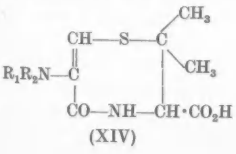
The specific radioactivity of penicillin synthesized in the presence of the compounds tested was very similar to that in control experiments with L-[*carboxy*-^{14}C]valine alone (column 5) and no marked decrease was observed. The low specific radioactivity of penicillin in one flask of Expt. no. 5 is not regarded as significant, particularly since penicillin from the duplicate flask had a slightly higher radioactivity than that in the control flasks. In Expt. no. 7 one of the two flasks also gave a low yield of penicillin and this result has been omitted from the comparison of the specific radioactivity of penicillin with that of the control (column 5). In both experiments, however, the results omitted would have given higher values for the mean specific radioactivity of penicillin than those given in column 5, and thus would not invalidate the general conclusion that addition of the test compounds did not decrease significantly the specific radioactivity of the synthesized penicillin.

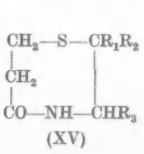
Biological properties of the peptides. The possible antibiotic activity of the cyclic peptides (III) and (IV) was investigated by the cup-plate bioassay with *Bacillus subtilis* (Humphrey & Lightbown, 1952). No zones of inhibition were obtained with concentrations of 1 mg./ml. The phenylacetyl peptide (IV) has also been tested as a possible inducer of penicillinase, but was found to be inactive (Pollock, 1957).

DISCUSSION

Synthesis and properties of the cyclic peptides

Several cyclic peptides of a type similar to those synthesized in the present work have been reported previously in the literature, but cyclization has often been brought about by ring-closure of the thio ether rather than of the peptide bond. Thus Süs (1948) prepared the peptide (XIV; $R_1=H$; $R_2=C_6H_5\cdot CH_2\cdot CO$) by interaction of phenacetylpenicillamine with ethyl orthoformate, and Knunyants & Linkova (1955) converted *N*-β-chloropropionylpenicillamine into (XV, $R_1=R_2=CH_3$; $R_3=CO_2H$) by hot methanolic baryta. However, attempts by Johnson (1956) to cyclize benzyloxycarbonyl-β-chloroalanylpenicillamine methyl ester to the benzyloxycarbonyl peptide (V) by a similar method gave the desired product only once and in poor yield (about 15%). This preparation could apparently not be repeated, and a comparison of the m.p. (190–210°) with that found in the present work (204°) suggests that the product may well have contained considerable amounts of an impurity.


(XIV)


(XV)

Two other types of syntheses have also been reported: Sheehan & Cruickshank (1956*a*, *b*) obtained the methyl ester of (XIV; $R_1R_2=$ phthaloyl; or $R_1=H$, $R_2=$benzylsulphonyl) as by-products in the lactamization of the corresponding derivatives of 2-aminocarboxymethyl-4-methoxycarbonyl-5:5-dimethylthiazolidine, and the unsubstituted compound (XV; $R_1=R_2=R_3=H$) has been prepared by ring enlargement of 1-thiatetrahydro-γ-pyrone oxime (Panizzi, 1948).

In the present work, the cyclic cysteinylvaline peptides were obtained by cyclization of suitably protected monoazides of ββ-dimethyl-lanthionine, as outlined in Fig. 1. Synthesis of the acetyl peptide (III) was carried out first because the starting materials were most easily available. It was also hoped that the final product (III) might be deacetylated to the amino peptide (VI) without rupture of the peptide bond. The latter could undoubtedly be converted into the phenylacetyl derivative (V) without undue difficulty, but selective removal of the acetyl group from (III) proved impracticable. The phenylacetyl peptide (IV) which resembles penicillin most closely was prepared next for tests as a penicillin precursor. When this compound was found to be inactive it was decided to synthesize also the free amino peptide (VI), which involved preparation of the required intermediates protected by the benzyloxycarbonyl group, which is easily removed by mild acid hydrolysis (Ben-Ishai & Berger, 1952).

The final cyclization of the azide, which took place in unexpectedly high yields, was based on the method previously used by Sheehan & Richardson (1954) for the preparation of cyclic 'triglycine', although it is now known that this product was in fact the dimeric cyclic hexaglycine (Bamford & Weymouth, 1955; Sheehan, Goodman & Richardson, 1955). In the present case, however, molecular-weight determinations of the peptides, as well as the absence of products other than monoacetyldimethyl-lanthionine (VII) when the acetylpeptide (III) was hydrolysed by acid under mild conditions,

indicate that dimerization to a 14-membered peptide did not occur to any significant extent. Molecular models show that the ring system in these peptides, unlike that in penicillin, is not strained because buckling of the ring is possible. On the other hand, the facile cleavage of the peptide bond by mild acid hydrolysis of (III) to (VII), and of (IV) to (VIII) (shown by paper chromatography only), suggests that this bond is unusually labile, particularly since cystinylvaline is completely hydrolysed by 6N-hydrochloric acid at 105° only after 72 hr. (H. R. V. Arnstein and D. Morris, unpublished experiments). It seems probable, therefore, that the seven-membered ring in the cyclic peptides is not entirely free of strain.

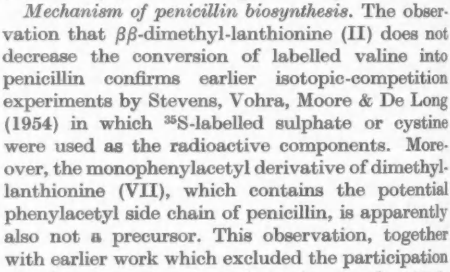
Penicillin biosynthesis

Possible precursor function of cyclic cysteinylvaline peptides and derivatives of dimethyl-lanthione. The results in Table 2 show clearly that none of the added peptides or thio ethers related to cystine and valine were utilized for penicillin biosynthesis in preference to L-valine, since this would have resulted in some decrease in the specific radioactivity of the penicillin. Although it is difficult to estimate quantitatively the change in radioactivity to be expected in such isotopic competition experiments, an approximation can be made on the basis of the following calculation. Since addition of labelled L-valine at a concentration of 50 mg./l. gives rise to penicillin having about 30–50 % of the molar radioactivity of the added valine (Arnstein & Clubb, 1957), the total metabolic 'pool' of valine and penicillin intermediates is approximately 2–3 times that of the added valine, i.e. 1–1·5 m-moles/l. In the present experiments, the unlabelled compounds were added at a level of 250 mg./l. (0·7–1·1 m-moles/l., according to the actual compound tested) and the specific radioactivity of penicillin synthesized in the presence of the unlabelled precursor would therefore be expected to be not greater than $100 \times 1{\cdot}5/(0{\cdot}7 + 1{\cdot}5) = 67$ % that in the control experiment without unlabelled precursor. In this calculation, however, no allowance has been made for the presence of two asymmetric centres in the cyclic peptides and dimethyl-lanthione derivatives; since only racemic products were used in this work the concentration of the correct stereoisomer will be only 25 % of the above figure, i.e. 0·2–0·3 m-mole/l. It would seem possible, therefore, that the specific radioactivity of penicillin produced could be as high as $100 \times 1{\cdot}5/(1{\cdot}5 \times 0{\cdot}2)$, i.e. 90 % of the control. It should be emphasized, however, that this represents the least favourable case; the metabolic 'pool' of an intermediate between valine and penicillin must be less than that of valine, since the last-named participates in the biosynthesis of products other than penicillin, such as proteins.

On the most favourable assumption, on the other hand, the metabolic 'pool' of an intermediate of penicillin biosynthesis may be considered to be of the same order of magnitude as the amount of penicillin produced, i.e. about 7·3 μg. (12 i.u.)/ml. (16 μmoles/l.) in the present experiments. The addition of 200 μmoles/l. of unlabelled intermediate to such a system would result in a marked decrease in the specific radioactivity of the penicillin, the expected radioactivity being $100 \times 16/(16 + 200)$, i.e. 7·5 % of that of the control.

In practice neither of these extreme situations would be expected to apply and a reasonable assumption would be that utilization of the unlabelled compounds tested in the present work should result in a 20–25 % decrease in the specific radioactivity of the penicillin. Effects of this magnitude have been observed in similar isotope-competition experiments with ^{35}S-labelled sulphate in which L-cysteine was shown to be a penicillin precursor (Stevens, Vohra, Inamine & Roholt, 1953), although both labelled and unlabelled compounds were added at higher concentrations (3 m-moles/l.) than in the present experiments.

Mechanism of penicillin biosynthesis. The observation that ββ-dimethyl-lanthionine (II) does not decrease the conversion of labelled valine into penicillin confirms earlier isotopic-competition experiments by Stevens, Vohra, Moore & De Long (1954) in which ^{35}S-labelled sulphate or cystine were used as the radioactive components. Moreover, the monophenylacetyl derivative of dimethyl-lanthionine (VII), which contains the potential phenylacetyl side chain of penicillin, is apparently also not a precursor. This observation, together with earlier work which excluded the participation of phenylacetylcystine (Arnstein *et al.* 1954), indicates that neither of the following alternative pathways is involved in penicillin formation:



The first step in penicillin biosynthesis therefore probably involves synthesis of the peptide bond with formation of either cystinyl- or cysteinyl-valine. Since both the cyclic peptide (VI) and its phenylacetyl derivative (IV) are apparently excluded as intermediates, subsequent steps may consist of synthesis of the β-lactam ring followed by closure of the thiazolidine thio ether bond, for example by a mechanism similar to that suggested by Arnstein & Crawhall (1957). Although there is at present no direct evidence for such a pathway,

none of the present or earlier results is inconsistent with such a mechanism, which may thus serve as a working hypothesis for future experiments.

SUMMARY

1. The synthesis of 6-amino-2:2-dimethyl-5-oxohexahydro-1:4-thiazepine-3-carboxylic acid (cyclic DL-cysteinyl-DL-valine) and its acetyl and phenylacetyl derivatives is described.

2. The monoacetyl and monophenylacetyl derivatives of $\beta\beta$-dimethyl-lanthionine, *S*-(2-acetamido-2-carboxyethyl)-DL-penicillamine and *S*-(2-phenylacetamido-2-carboxyethyl)-DL-penicillamine respectively have also been prepared.

3. The compounds were tested as penicillin precursors by an isotope-competition method in which washed mycelium of *Penicillium chrysogenum* WIS 51–20 was incubated with L-[*carboxy*-^{14}C]valine and the effect of addition of the unlabelled compounds on the specific radioactivity of the penicillin produced was measured.

4. None of the compounds was found to be an intermediate in the conversion of valine into penicillin. Phenylacetyl-L-cysteinyl-D-valine and $\beta\beta$-dimethyl-lantionine were also inactive as penicillin precursors.

5. Possible mechanisms of penicillin biosynthesis are discussed.

We wish to thank Dr K. Folkers, Merck and Co. Inc., for a gift of phenylacetyl-L-cysteinyl-D-valine.

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The Metabolism of C_2 Compounds in Micro-organisms

1. THE INCORPORATION OF [2-^{14}C]ACETATE BY *PSEUDOMONAS FLUORESCENS*, AND BY A *CORYNEBACTERIUM*, GROWN ON AMMONIUM ACETATE

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During the growth of micro-organisms, intermediates of the tricarboxylic acid cycle are utilized to provide the carbon skeletons of synthesized amino acids. When such growth occurs on complex substrates, carbon dioxide-fixing reactions, such as form oxaloacetate or malate from pyruvate, are thought to replenish the supply of tricarboxylic acid cycle intermediates (Wiame & Bourgeois, 1955). Such reactions are not, however, sufficient to explain the processes by which micro-organisms

can grow on simple substrates, such as ethanol or acetate, as sole carbon sources. The tricarboxylic acid cycle is a means whereby acetate, after reacting with oxaloacetate, is oxidized to carbon dioxide, with the regeneration of oxaloacetate. Any removal of intermediates from the cycle would prevent the regeneration of oxaloacetate, and would therefore cause the cycle to stop. Mechanisms must therefore operate under these conditions whereby the acceptor for C_2 units can be synthesized from acetate.

The present work reports evidence for the operation of such a mechanism in acetate-grown *Pseudomonas fluorescens* KB1 (Kogut & Podoski, 1953) and in a bacterium tentatively identified as *Corynebacterium* sp. The findings are in accordance with the operation in these organisms of the 'glyoxylate by-pass' of the tricarboxylic acid cycle, demonstrated in cell-free extracts (Kornberg & Madsen, 1957*a*, *b*; Kornberg & Krebs, 1957). This work has been presented in part to the Biochemical Society (Kornberg, 1957*a*).

METHODS

Maintenance and growth of organisms. Cultures of both *Pseudomonas* KB1 (Kogut & Podoski, 1953) and an airborne Gram-positive organism tentatively identified as *Corynebacterium* sp. were maintained on slopes consisting of 25 mM-ammonium acetate, 25 mM-sodium potassium phosphate buffer, pH 7·1, essential salts (2 mg. of $CaCl_2,6H_2O$, 0·2 mg. of $MnSO_4,4H_2O$, 4 mg. of $MgSO_4,7H_2O$ and 0·2 mg. of $FeSO_4,6H_2O$/100 ml. of medium) and 2% (w/v) of agar agar (Hopkin and Williams Ltd., Chadwell Heath, Essex). Stock cultures of the organisms were subcultured every 2 weeks, grown at 30° and stored at 2°.

For growth in liquid medium, a loopful of organisms from a freshly grown slope was suspended in a Carrel culture flask (J. A. Jobling and Co. Ltd., Sunderland) containing 400 ml. of a mixture of 50 mM-ammonium acetate, 50 mM-phosphate buffer, pH 7·1, and essential salts of the same concentration as those used in the solid medium. The flasks were shaken for 10–12 hr. at 30° whilst partially immersed in a Warburg bath. Growth was determined by measurements in an EEL nephelometer (Evans Electroselenium Ltd., Harlow, Essex) of the light-scattering of samples of the bacterial suspensions and comparison of the observed reading with a previously constructed calibration curve relating such readings to the dry weight of bacteria/ml. of suspension. Although the medium was adequate to sustain growth of over 1 mg. dry wt./ml., the bacteria were removed whilst still in the phase of logarithmic growth, at densities of 0·2–0·3 mg. dry wt./ml. The contents of the Carrel flasks were centrifuged at 10° for 15 min. at 1500 *g*.

Manometric experiments. For measurements of the oxidation of acetate and of tricarboxylic acid cycle intermediates, the centrifuged bacteria were suspended in 25 mM-phosphate buffer, pH 7·1, and re-centrifuged. The washed bacteria were resuspended in 25 mM-phosphate buffer, pH 7·1, to a final concentration of 2 mg. dry wt. of bacteria/ml. The rate of O_2 uptake on addition of substrates was measured with Warburg manometers. Flasks (approx. vol., 25 ml.) with single side arms contained 1 ml. of the bacterial suspension, 0·1 ml. of water and 0·5 ml. of 0·2M-phosphate buffer, pH 7·1, in the main compartments, and 20 μmoles of substrates in the side arms. Strips of Whatman no. 40 filter paper and 0·2 ml. of 2N-KOH were placed in the centre wells. The manometers were gassed with air, equilibrated at 30° for 10 min. and the contents of the side arms mixed with those of the main compartments. Readings were taken at 5 min. intervals, and recorded as the total O_2 absorbed in μl.

Purification of [2-^{14}C]*acetate.* Sodium [2-^{14}C]acetate, obtained from the Radiochemical Centre, Amersham, Bucks, was found to contain small but significant amounts of propionate and of non-volatile impurities, which increased with time and therefore presumably arose by radiation decomposition. For purification of acetate, the contents of the sealed phials were dissolved in water and chromatographed as strips on Whatman no. 3 paper by the method of Kennedy & Barker (1951). The papers, after chromatography, were heavily sprayed with 0·5M-K_2HPO_4, air-dried and the acetate area located by radioautography. This area was eluted with 0·05N-NaOH into a 100 ml. Pyrex round-bottomed flask fitted with a B19 standard ground-glass joint. The flask was connected to a large transfer unit (Kornberg, Davies & Wood, 1952) and a receiver attached to the other end. The contents of the flask were frozen in liquid N_2, the unit was evacuated with an oil pump and the tap closed. The flask contents were now allowed to thaw whilst the receiving flask was cooled in liquid N_2. Water rapidly distilled over at room temperature into the receiver, leaving a mixture of alkaline acetate and phosphates in the distilling flask. When the volume had been reduced to 0·5–1 ml., the vacuum was released and the receiving flask discarded. The contents of the distilling flask were frozen in liquid N_2 and 0·5 ml. of 5N-H_2SO_4 was pipetted on to the frozen solution. A long thin flask, containing a slight excess of KOH, was connected to the receiving end of the transfer unit, the system evacuated and the tap closed. The receiver was cooled in liquid N_2 and the contents of the distilling flask were allowed to thaw. As the ice melted, labelled acetic acid was liberated and, with accompanying water, rapidly distilled at room temperature into, and condensed in, the cooled receiver. After 20–30 min., distillation was complete and the distilling flask contained only oily H_2SO_4. At this time, the tap was opened, the receiving flask detached and stoppered. When the contents of this flask thawed, the KOH present neutralized the labelled acetic acid to a pH of approx. 9. The solution was adjusted to pH 7·4 and stored at −12°. Chromatographic analysis showed that acetate was the only volatile acid present, and that the solution, freshly purified or stored at −12° for up to 3–4 weeks, contained no non-volatile impurities.

Bacterial incorporation of [^{14}C]*acetate.* Bacteria, which had been centrifuged after growth in Carrel culture flasks, were suspended without washing in fresh growth medium containing 25 mM-ammonium acetate, 25 mM-phosphate buffer and essential salts. The concentration of bacteria was adjusted to be 0·1–0·2 mg. dry wt./ml. Of this suspension, 19 ml. was pipetted into the flask of an automatic pipette (Bartley, 1954) and shaken at 30° with partial immersion in a Warburg bath. (Pipettes of this type are available from Exelo Products, W. G. Flaig and Sons, Ltd., 39 Waterloo Road, London, N.W. 2.) Samples (3 ml.) were

removed after 30, 60 and 90 min. and the density of bacteria was measured nephelometrically. When logarithmic growth had been established (mean generation time 1·25 hr.), the flask was removed from the bath, 1 ml. of the [^{14}C]acetate solution (containing 41 μmoles of acetate and giving $1{\cdot}4 \times 10^7$ counts/min. under the conditions of radioassay employed) was added and the top of the pipette replaced. After rapid manual swirling of the contents, to ensure good mixing, samples (1 ml.) were pipetted into thick-walled Pyrex (16 mm. × 125 mm.) tubes, each containing 3 ml. of absolute ethanol, standing in a bath at 80°. The times of removal of samples were measured with a stop watch, which had been started at the time of addition of the isotopic solution.

Treatment of aqueous ethanolic suspensions. The hot ethanolic (75%) suspensions were left at 50° for 15 min., cooled and centrifuged at 1500 *g*. The clear supernatant solutions were poured into similar thick-walled Pyrex tubes and the precipitates resuspended each in 1 ml. of 20% (v/v) aqueous ethanol. After re-centrifuging, the supernatant solutions were combined and evaporated to dryness at 60° under a stream of H_2SO_4-washed N_2. The precipitates were suspended in 1 ml. of 6N-HCl, the tubes containing these suspensions sealed in an O_2 flame and the tubes left at 110° for 20 hr. After cooling, the tubes were opened and evaporated to dryness at 100° under a stream of H_2SO_4-washed N_2.

Chromatographic analysis. The dry material obtained from the ethanol-soluble or ethanol-insoluble portions of the bacterial samples was suspended in 0·5 ml. of 20% (v/v) aqueous ethanol and centrifuged if necessary. Samples (250 μl.) of the clear supernatant solutions were applied to the 'origin' positions (3 in. from each side of one corner) of sheets of Whatman no. 4 chromatographic paper (20 in. × 18 in.). After equilibration the papers were analysed by descending chromatography in phenol–formic acid (500 g. of phenol:13 ml. of 90% HCO_2H:167 ml. of water) in the short direction. The papers were air-dried for 24 hr., equilibrated with the second solvent and chromatographed in the second direction in the butanol–propionic acid–water solvent of Calvin & Benson (1949). Acetate and other volatile compounds were removed by this procedure.

In both directions, the solvents were allowed to flow to within 1 in. from the edge. Good separations were achieved of most of the labelled compounds formed.

Radioautography and radioassay. The air-dried chromatograms were marked at the corners and at the solvent fronts with indian ink, which had been rendered radioactive by addition of waste samples of ^{14}C-labelled fractions, and were placed in lead-backed X-ray holders, 14 in. × 17 in. (Kodak Ltd.). A sheet of Standard or Blue Brand X-ray film (Kodak Ltd.) was applied to each chromatogram and the closed holders were stored for 3–14 days under a pile of similar holders, efficient contact between films and chromatograms thus being ensured. By superimposing the radioactive-ink markings on the developed films and on the chromatograms, the positions of labelled compounds were accurately located. These labelled areas were outlined in pencil on the chromatograms and their radioactivity was measured by direct radioassay on the papers with a mica end-window β-counter tube (General Electric Co., type 2B2) standing on a Perspex mask into which a square (3·2 cm. × 3·2 cm.) had been cut. The radioactive areas were thus counted completely without danger of overlapping of spots. Each area was counted for at least 3000 counts total, giving a standard error of radioassay of less than ±2%, and the observed counts were corrected for background radioactivity.

Identification of labelled compounds. The labelled materials, which had been approximately identified in the two solvents used, were eluted, mixed with unlabelled compounds and rechromatographed in the usual and at least two other solvents. Carboxylic acids were thus further analysed by chromatography in butanol–water–diethylamine (Jones, Dowling & Skraba, 1953) and in pentanol–aq. 5M-formic acid (Buch, Montgomery & Porter, 1952). Amino acids were chromatographed in the solvents *B*, *E* and *O* of Hardy, Holland & Nayler (1955). Phosphorylated compounds were analysed by ascending chromatography in ethanol–acetic acid–water (75 ml. of 95% ethanol:2 ml. of acetic acid:23 ml. of water), and by descending chromatography in water-saturated phenol.

Both the position of isotopic material, revealed on film by radioautography and on the chromatograms by the appropriate spray, and the outlines of the markings, had to correspond exactly before identity of the labelled and unlabelled material was taken to be established.

Sprays. Unlabelled carboxylic acids were detected on chromatograms by spraying with 0·04% (w/v) ethanolic bromophenol blue, adjusted to pH 8 with NaOH; amino acids with 0·4% (w/v) ninhydrin in water-saturated butanol, and phosphorylated compounds by the spray and procedure of Hanes & Isherwood (1949).

RESULTS

Oxidation of tricarboxylic acid cycle intermediates

Washed suspensions of acetate-grown *Pseudomonas* KB1 oxidized acetate and all the intermediates of the tricarboxylic acid cycle (Fig. 1). The rates of oxygen uptake were highest with acetate, which had a Q_{O_2} value (μl. of O_2/mg. dry wt./hr.) of 120–140, and the α-oxo acids, α-oxoglutarate and oxaloacetate. No lag was observed in the oxidation of malate. A lag of approx. 20 min. was noted with succinate and fumarate, and approx. 30 min. with *iso*citrate. Citrate was oxidized after a lag of 50–60 min. It is probable that these delays, noted also with *P. fluorescens* grown on acetate (Campbell & Stokes, 1951), fumarate (Barrett, Larson & Kallio, 1953) or succinate (Kogut & Podoski, 1953) as sole carbon sources, are associated with the elaboration of mechanisms for the transport of substrates into the cell (Davis, 1956).

Utilization of labelled acetate. When [2-^{14}C]acetate was added to growing suspensions of *Pseudomonas* KB1, isotope was incorporated into both the ethanol-soluble and the protein fractions (Fig. 2). The incorporation of radioactive carbon into the soluble fraction was linear with time over the duration of the experiment (3·25 min.).

Distribution of ^{14}C amongst the labelled constituents of the soluble fractions. Two-dimensional chromatography and radioautography of the ethanol-soluble fractions obtained from each sample of the

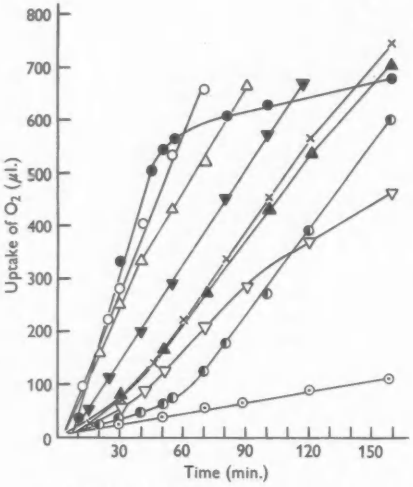

Fig. 1. Oxidation of tricarboxylic acid cycle intermediates by *Pseudomonas* KB1. Manometer flasks contained washed bacteria (2 mg. dry wt.) in 78 mM-potassium phosphate, pH 7·1, in the main compartments, 20 μmoles of substrates (as indicated) in the side bulbs, and 400 μmoles of KOH in the centre well. Incubated in air at 30°; substrates were added at zero time. ●, Acetate; ○, oxaloacetate; △, α-oxoglutarate; ▼, malate; ×, succinate; ▲, fumarate; ▽, *iso*citrate; ◐, citrate; ⊙, endogenous respiration.

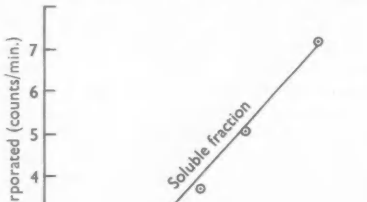

Fig. 2. Incorporation of ^{14}C from [2-^{14}C]acetate into the protein and ethanol-soluble fractions of *Pseudomonas* KB1 growing on acetate. Conditions were similar to those described in Table 1.

growing suspension showed that ^{14}C from acetate rapidly appeared in many of the intermediates of the tricarboxylic acid cycle and in amino acids, such as aspartate and glutamate, directly derived therefrom (Table 1). Even after only 3 sec., isotope could be detected in malate, citrate, succinate and aspartate. Glutamate became detectably labelled after this time, and progressively increased in radioactivity until it formed the predominant fraction of the labelled compounds present. Phosphorylated compounds, such as phosphopyruvate and the phosphoglycerates, did not become labelled until 26 sec. after addition of the labelled acetate, by which time a high level of labelling had been reached in other compounds. This showed that carbon dioxide-fixing mechanisms, such as operate in photosynthetic (Calvin, 1955) or autotrophic (Santer & Vishniac, 1955; Trudinger, 1955) organisms, were not of primary importance in the incorporation of acetate.

The procedure employed (rapid sampling with an automatic pipette delivering 1 ml. samples with a variation of ±0·1 ml. between successive samples) yielded results which are quantitatively only approximately accurate. Thus no emphasis can be placed on the absolute values of the incorporated radioactivities; between 18 and 33 sec., for example, only the glutamate area apparently increased in activity, whereas after this time major increases were noted in all compounds (Table 1). This phenomenon is inevitably associated with the incomplete delivery of samples from the automatic pipette under conditions of very rapid operation. These considerations do not, however, render the results obtained useless. Within each sample, the distribution of radioactivity amongst the various labelled compounds fairly reflects the course of incorporation of acetate. When the proportion of the total radioactivity of each sample contributed by each labelled compound, expressed as the percentage of the total ^{14}C in the sample, was plotted against time, smooth curves could be drawn through the points obtained (Fig. 3). Initially, over 40 % of the incorporated ^{14}C was present in malate and 30 % in citrate. Succinate and fumarate contained a further 10 % and aspartate approx. 11 %. These proportions rapidly altered with time; both malate and citrate decreased sharply, whereas aspartate, after a rise to over 30 %, decreased to a steady level at which it contributed some 18 % to the total radioactivity of each sample. After 30 sec., a 'steady state' had been reached. At and after this time, the malate area contained approx. 10 % of the total radioactivity, citrate approx. 15 %, succinate plus fumarate 5 % and glutamate approx. 40 %. These steady levels may be taken to reflect also the 'pool sizes' of the various compounds, since, by this time, all these intermediates appear to be saturated with the isotope entering from the administered [^{14}C]acetate.

Since the fully-labelled malate 'pool' in the steady state was lower in radioactivity than the

Table 1. *Distribution of radioactive carbon amongst the non-volatile labelled components of the ethanol-soluble fractions of growing suspensions of* Pseudomonas *KB*1 *incubated with potassium* [2-^{14}C]*acetate*

A solution of $^{14}CH_3 \cdot CO_2K$, containing 41 μmoles of acetate and 200 μc of ^{14}C, was added at zero time to 10 ml. of a suspension of bacteria, growing in an automatic pipette on 25 mM-ammonium acetate. Samples (approx. 1 ml.) were pipetted at known times into 3 ml. of hot absolute ethanol. The precipitates were each washed with 1 ml. of 20% (v/v) aqueous ethanol. Portions of the combined supernatant solutions were evaporated to dryness under N_2, resuspended in 20% (v/v) aqueous ethanol and analysed by two-dimensional paper chromatography. The radioactive compounds were located by radioautography, and the ^{14}C content was determined by direct radioassay of the radioactive areas with a mica end-window β-counter tube.

	Radioactivity (counts/min.) in							
Time (sec.)	Malate	Citrate and *iso*citrate	Fumarate and succinate	Aspartate	Glutamate	Phosphorylated compounds	Other compounds	Total
3	21	15	5	6	0	0	3	50
8	42	28	14	39	13	0	4	140
12	49	40	15	68	73	0	7	252
18	133	142	48	209	390	0	18	940
26	133	164	62	195	443	20	34	1056
33	132	154	60	192	492	34	38	1102
48	244	273	116	312	760	78	67	1850
60	216	312	104	375	832	208	133	2180
79	272	296	122	464	964	360	198	2676

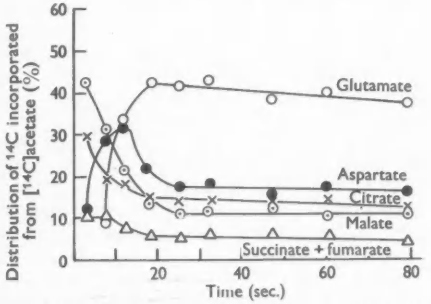

Fig. 3. Variation with time of the percentage distribution of ^{14}C from [2-^{14}C]acetate incorporated into the constituents of the ethanol-soluble fraction of *Pseudomonas* KB1 growing on acetate. Conditions were similar to those described in Table 1.

citrate 'pool', the initially higher labelling of the malate area shows that, in the earliest stages of the incubation, isotope from acetate entered malate without passing through the stage of citrate. This therefore indicates that acetate entered the tricarboxylic acid cycle at at least two sites, to form citrate at one, and a compound in ready isotopic equilibrium with, or identical with, malate at another. This conclusion is confirmed when the total radioactivity in the combined citrate plus glutamate fraction, and that in the C_4 compounds, is plotted against time (Fig. 4). The relative 'pool' sizes in the steady state, reached after 30 sec., show that the C_4 compounds contain approx. 35% less isotope than does the citrate plus glutamate 'pool'. Initially, however, this position is reversed: at the earliest stages, the activity of the C_4 compounds is more than double that of the citrate plus glutamate area. This shows that isotope from acetate, appearing in the C_4 compounds, cannot exclusively have come via the stage of citrate, but must also have entered the tricarboxylic acid cycle by a route not involving a condensation of acetate and oxaloacetate to form citrate. The low initial radioactivity of phosphorylated compounds, shown in Fig. 4, again emphasizes that these substances do not play an essential role in the initial formation of C_4 compounds from acetate.

Experiments with Corynebacterium *sp.* Results substantially similar to those obtained with *Pseudomonas* KB1 were obtained with a Gram-positive organism, isolated from a vessel containing the 25 mM-ammonium acetate medium, which had been left exposed to the air for some days. (This organism was isolated in pure culture by Mr W. E. Patey, and experiments on it were performed in collaboration with him. It was tentatively identified as *Corynebacterium* sp. by Dr D. E. Hughes.)

This organism grew more slowly than did *Pseudomonas* KB1 (mean generation time 3·5 hr.). The percentage distributions of isotope from [2-^{14}C]acetate (Fig. 5) were therefore inevitably different from those shown in Fig. 3. The citrate 'pool' contained relatively more isotope, and the increase in the label of the glutamate, derived from it, was less striking than was observed with *Pseudomonas* KB1. Similarly, the succinate and fumarate areas were relatively more radioactive. Yet, when the total radioactivities of the citrate plus glutamate, and the C_4 compounds, were plotted against time (Fig. 6), curves strikingly similar to those shown in Fig. 4 were obtained.

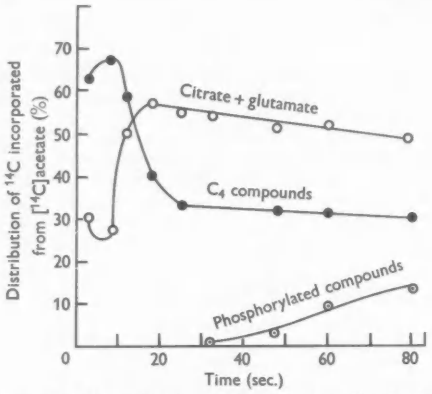

Fig. 4. Variation with time of the percentage distribution of ^{14}C from [2-^{14}C]acetate incorporated into some components of the ethanol-soluble fraction of *Pseudomonas* KB1, growing on acetate. Conditions were similar to those described in Table 1.

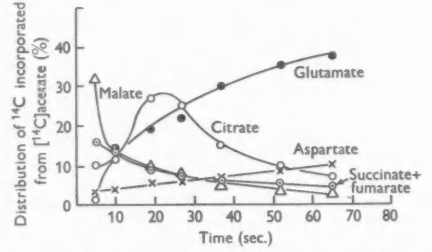

Fig. 5. Variation with time of the percentage distribution of ^{14}C from [2-^{14}C]acetate incorporated into the constituents of the ethanol-soluble fraction of *Corynebacterium* sp. growing on acetate. Conditions were similar to those described in Table 1.

Again the percentage labelling of the citrate plus glutamate area was higher than that of the C_4 compounds after establishment of the 'steady state', but much lower in the initial stages of the incubation. These results therefore show that in this organism, as in *Pseudomonas* KB1, isotope from acetate was initially introduced into C_4 compounds without passing through the stage of citrate.

DISCUSSION

When micro-organisms grow on acetate as sole source of carbon, intermediates are drained from the tricarboxylic acid cycle to provide the carbon skeletons of amino acids, and net syntheses of C_4 dicarboxylic acids must occur to provide the acceptor for C_2 units entering the cycle. These net

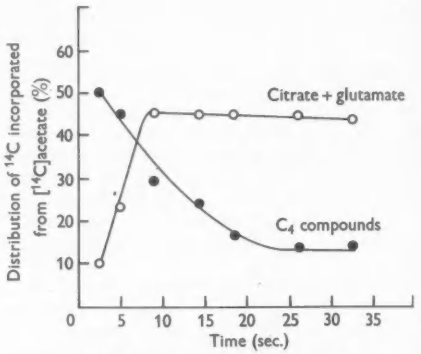

Fig. 6. Variation with time of the percentage distribution of the ^{14}C from [2-^{14}C]acetate incorporated into some components of the ethanol-soluble fraction of *Corynebacterium* sp. growing on acetate. Conditions were similar to those described in Table 1.

syntheses can be explained in a number of ways. An oxidation of acetate to carbon dioxide, and subsequent fixation of carbon dioxide by the mechanism operating in photosynthetic (Calvin, 1955) and autotrophic (Santer & Vishniac, 1955; Trudinger, 1955) organisms, might occur. This is, however, rendered unlikely by the finding (Fig. 4) that phosphorylated compounds do not become labelled until other compounds contain relatively high levels of radioactivity. Similar objections apply to the suggestion that a fixation of carbon dioxide by acetate, to give phosphopyruvate (Abelson, Bolton, Britten, Cowie & Roberts, 1954) could supply the necessary intermediates. Studies of the incorporation of carbon dioxide by *Pseudomonas* KB1 growing on ammonium acetate showed that a compound in ready equilibrium with carbon dioxide does lie on the pathway of acetate (Kornberg, 1956), but that the total quantity of carbon fixed from carbon dioxide is only 15–17% of that incorporated from acetate (Kornberg, 1957*b*). These findings, and the results of degradative studies on the location within the molecules of synthesized amino acids of carbon derived from added carbon dioxide (Kornberg & Quayle, 1958), rule out carbon dioxide fixation as being of primary importance in the incorporation of acetate.

A direct condensation of two molecules of acetate to give succinate, first postulated by Thunberg (1920), would similarly supply necessary C_4 compounds. There is no evidence for the occurrence of this reaction. Although Seaman & Naschke (1955) have recently claimed a direct formation of succinate from acetyl-coenzyme A to occur in *Tetrahymena* and *Escherichia coli*, the present results show that this reaction plays no

major role in *Pseudomonas* KB1. After brief incubation of *Pseudomonas* KB1 with labelled acetate, only small amounts of ^{14}C were incorporated into succinate. A direct formation of succinate from the condensation of two molecules of acetyl-coenzyme A could not have given the results of Table 1. Similarly, no evidence for the direct formation of succinate from acetyl-coenzyme A was obtained with cell-free extracts (Kornberg & Madsen, 1957*a*, *b*).

The results given in this paper show that acetate enters the tricarboxylic acid cycle at at least two sites, to form citrate at one and a compound identical, or in close equilibrium, with malate at the other. Investigations of cell-free extracts of *Pseudomonas* KB1 (Kornberg & Madsen, 1957*a*, *b*) support this conclusion. Acetyl-coenzyme A has been shown to react both with oxaloacetate, to give citrate, and glyoxylate (derived from the aldol fission of *iso*citrate) to give malate. The net formation of malate and succinate from acetate and *iso*citrate (Kornberg & Madsen, 1957*a*, *b*) under the action of *iso*citritase (Smith & Gunsalus, 1954; Saz & Hillary, 1956) and malate synthetase (Wong & Ajl, 1956) would result in the net synthesis of C_4 dicarboxylic acids. The present results are in agreement with the operation of this mechanism *in vivo*, termed the 'glyoxylate cycle' (Kornberg & Krebs, 1957; Kornberg & Madsen, 1958), which has been demonstrated *in vitro*. Such a mechanism, utilizing the intermediates of the tricarboxylic acid cycle, represents a modification of that cycle leading to a net synthesis of cell constituents from C_2 units and appears to be of importance not only in the growth of micro-organisms on acetate, but also in the metabolism of fungi (Kornberg & Krebs, 1957) and in conversions of fat into carbohydrate (Kornberg & Beevers, 1957; Callely, Dagley & Hodgson, 1957).

SUMMARY

1. Washed cells of *Pseudomonas fluorescens* KB1, grown on ammonium acetate as sole carbon source, rapidly oxidize acetate and all the intermediates of the tricarboxylic acid cycle, confirming that this cycle operates in this organism.

2. Cultures of *Pseudomonas* KB1 and of a Gram-positive organism tentatively identified as *Corynebacterium* sp., growing on ammonium acetate, were incubated for short periods (3 sec.–5 min.) with [2-^{14}C]acetate. Isotope was incorporated into both the 75% aqueous ethanol-soluble, and into the protein, fractions.

3. The distribution of the incorporated isotope amongst the labelled compounds in the ethanol-soluble fraction showed that intermediates of the tricarboxylic acid cycle, and amino acids directly derived therefrom, rapidly became labelled.

4. Although the proportion of the total radioactivity present in citrate and glutamate was higher than that present in the C_4 dicarboxylic acids after establishment of isotopic 'steady-state' conditions, the distribution of radioactivity during the earliest stages of the incubation was radically different. Initially, over 70% of the total isotope incorporated was present in C_4 compounds.

5. These results show that, initially, the label was introduced into malate without passing through citrate, and that therefore a second point of entry of acetate into the tricarboxylic acid cycle must exist in these organisms.

6. These findings are in accordance with the operation in intact cells of the 'glyoxylate by-pass' of the tricarboxylic acid cycle, demonstrated in cell extracts. This by-pass serves to replace, from acetate, C_4 dicarboxylic acids drained from the tricarboxylic acid cycle when micro-organisms grow on this single carbon source.

I wish to thank Professor H. A. Krebs, F.R.S., for his interest and encouragement, Mr W. E. Patey for his collaboration in part of this work, Dr D. E. Hughes for his help in microbiological procedures and Dr M. Kogut for the gift of a culture of *Pseudomonas fluorescens* KB1. This work was sponsored by the Air Research and Development Command of the United States Air Force, through its European Office, under contract no. AF 61(514)–1180, and by the Rockefeller Foundation.

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The Metabolism of C_2 Compounds in Micro-organisms

2. THE EFFECT OF CARBON DIOXIDE ON THE INCORPORATION OF [^{14}C]ACETATE BY ACETATE-GROWN *PSEUDOMONAS* KB1*

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When micro-organisms grow on acetate as sole source of carbon, intermediates are drained from the tricarboxylic acid cycle and are utilized to provide the carbon skeletons of amino acids. Under these circumstances, net syntheses of C_4 dicarboxylic acids must occur from acetate to replace the acceptor for C_2 units entering the cycle. Experiments on acetate-grown *Pseudomonas* KB1 (Kornberg, 1958) showed that acetate enters the cycle at two sites, to form citrate at one and a compound metabolically close to malate at the other. It is known that a reductive condensation of carbon dioxide with pyruvate is one of the routes of biosynthesis of malate (Wood & Werkman, 1938; Ochoa, Mehler & Kornberg, 1948; Davis, Cheldelin, Christensen & Wang, 1956), and it has been postulated that in acetate-grown *Escherichia coli* pyruvate or phosphopyruvate can arise from a condensation of carbon dioxide with acetate (Abelson, Bolton, Britten, Cowie & Roberts, 1954). It is therefore feasible that the necessary net syntheses of C_4 dicarboxylic acids from acetate proceed via consecutive carboxylation reactions. Studies of the effect of unlabelled carbon dioxide on the incorporation of ^{14}C by *Pseudomonas* KB1 growing on [^{14}C]acetate were therefore undertaken on the assumption that the postulated reactions would be reflected in the quantity of carbon from carbon dioxide appearing in proteins and in the labelling patterns of synthesized amino acids.

The results of such experiments rule out the occurrence of condensations of C_2 units with carbon dioxide as being of quantitative significance in the biosynthesis of cell constituents from acetate, and show that 15–17% of the total carbon incorporated into protein from acetate is exchangeable with carbon dioxide. The observed labelling patterns are in good agreement with the occurrence in the growing cells of the 'glyoxylate cycle' (Kornberg & Madsen, 1957, 1958; Kornberg & Krebs, 1957) demonstrated in bacterial extracts.

* Part 1: Kornberg (1958).

METHODS

Growth of organisms. Pseudomonas fluorescens KB1 (Kogut & Podoski, 1953) was grown in Carrel culture flasks (J. A. Jobling and Co. Ltd., Sunderland), each containing 400 ml. of a liquid phosphate–acetate–salts medium, as previously described (Kornberg, 1958). The bacterial suspensions were centrifuged at 10° when growth had reached the mid-point of the logarithmic phase, and were resuspended in fresh growth medium at concentrations of 2–3 mg. dry wt./ml. for short-term incubations, and at 0·1–0·2 mg. dry wt./ml. for long-term experiments.

Isotopic materials. [^{14}C]Acetate, labelled in either the methyl or the carboxyl group, was obtained from the Radiochemical Centre, Amersham, Bucks., and was dissolved in water to contain 200 μc of ^{14}C/ml. For the experiments with doubly-labelled acetate, equal volumes of each

of the solutions of $^{14}CH_3 \cdot CO_2Na$ and $CH_3 \cdot {}^{14}CO_2Na$ were mixed before use.

Brief incubations. Differences in the rates of incorporation of [^{14}C]acetate in the presence and in the absence of unlabelled carbon dioxide were studied in experiments in which 0·5 ml. of [^{14}C]acetate solution and 2·5 ml. of water, containing 100 μc of ^{14}C and 20·5 μmoles of sodium acetate, were added to one of a set of three similar flasks, each containing 3 ml. of a suspension of bacteria growing logarithmically on 25 mM-ammonium acetate. To the second flask was added the same amount of [^{14}C]acetate + 1·5 ml. of water + 1 ml. of 26 mM-$NaHCO_3$, and the third received [^{14}C]acetate + 2·5 ml. of 26 mM-$NaHCO_3$. Samples (1 ml.) were taken at known times from all flasks and pipetted into tubes containing 1 ml. of 10% (w/v) trichloroacetic acid. The precipitates were centrifuged, washed with 0·5 ml. of hot 5% (w/v) trichloroacetic acid, and suspended in 1 ml. of 6N-HCl. The tubes were sealed in the flame and kept at 110° for 18 hr. The protein hydrolysate solutions were evaporated to dryness under H_2SO_4-washed N_2, dissolved each in 0·5 ml. of water and analysed by two-dimensional chromatography and radioautography as previously described (Kornberg, 1958).

Prolonged incubations. For long-term studies of the effect of unlabelled carbon dioxide on the incorporation of [^{14}C]acetate into synthesized amino acids, bacteria were suspended in two flasks: one (flask *A*) contained 15 ml. of a medium containing 50 mM-ammonium acetate, 50 mM-phosphate, pH 7·0, and salts (Kornberg, 1958), whereas the other (flask *B*) contained in addition 100 mM-$NaHCO_3$. Flask *A* was gassed with 100% O_2 and stoppered tightly, and *B* was gassed continuously with $CO_2 + O_2$ (5:95). Both were incubated at 22° or 30°, and growth was followed by nephelometric determinations of cell density. When logarithmic growth had been established, flask *A* was gassed again with 100% O_2 and [^{14}C]acetate solution (1 ml., containing 200 μc of ^{14}C, and either 41 μmoles of $^{14}CH_3 \cdot CO_2Na$ or 18 μmoles of $^{14}CH_3 \cdot {}^{14}CO_2Na$) was added to each flask, *A* then being tightly stoppered whilst gassing with $CO_2 + O_2$ (5:95) was continued in *B*. Samples (1–2 ml.) were removed at known times and pipetted into equal volumes of 10% (w/v) trichloroacetic acid. The protein precipitates were washed, hydrolysed and analysed as described for short-term incubation experiments.

Measurement of synthesized protein. Besides nephelometric determinations of cell density throughout the duration of long-term experiments, the amino acid N content of each sample of hydrolysed protein was determined by a modification of the quantitative ninhydrin reaction (Kornberg & Patey, 1957).

Purification and degradation of amino acids. Samples of labelled amino acids, which were derived from the protein hydrolysates obtained in short- or long-term experiments and which had been located on chromatograms by radioautography, were eluted with water and rechromatographed in ethanol–butanol–water (10:10:5, by vol.; Hardy, Holland & Nayler, 1955). The eluted amino acid was purified by co-crystallization with an authentic sample of the unlabelled amino acid until constancy of specific radioactivity, established by total combustion with Van Slyke–Folch reagent (Van Slyke & Folch, 1940) and radioassay of $Ba^{14}CO_3$, had been achieved. The following procedures were used for degradation of individual amino acids.

Alanine was decarboxylated with ninhydrin and the resulting $^{14}CO_2$, from the carboxyl carbon, was trapped in alkali and counted as $Ba^{14}CO_3$. The acetaldehyde was trapped in 2% (w/v) sodium bisulphite solution and oxidized to acetic acid by chromic acid at room temperature (Sakami, 1955). The acetic acid was converted into $^{14}CO_2$ and methylamine by Schmidt degradation (Phares, 1951); the methylamine was burnt, in the form of methylamine sulphate, to $^{14}CO_2$ and counted as $Ba^{14}CO_3$.

Aspartic acid was decarboxylated with ninhydrin and the resulting $^{14}CO_2$ from both carboxyl groups counted as $Ba^{14}CO_3$. The determination of the radioactivity of individual carboxyl groups was made by decarboxylation with chloramine-T (Ehrensvärd, Reio, Saluste & Stjernholm, 1951).

Assays of radioactivity. The ^{14}C content of amino acids on chromatograms was determined, by direct radioassay on the papers of spots located by radioautography, with a mica end-window β-counter tube (G.E.C., type 2B2).

Samples of $Ba^{14}CO_3$, prepared in triplicate, were counted with a lead-shielded mica end-window counter and corrected for self-absorption to infinite thinness. All counts were corrected for background. A total of at least 3000 counts was recorded for each spot on chromatograms (standard error of counting of less than $\pm 2\%$) and of at least 5000 counts (standard error of less than $\pm 1\%$) for each sample of $Ba^{14}CO_3$.

RESULTS

Short-term incubations

Comparison of the rate of incorporation of ^{14}C from [2-^{14}C]acetate and from unlabelled acetate and [^{14}C]bicarbonate (Kornberg, 1956) shows that about one-eighth as much isotope was incorporated from bicarbonate as from acetate. This figure was a minimum one; isotope from the added bicarbonate had to enter metabolic 'pools' against a stream of unlabelled CO_2, produced by the oxidation of the unlabelled acetate. In order to arrive at a more accurate estimate of the extent of fixation of CO_2, use was made of the isotope-competition technique of Roberts, Abelson, Cowie, Bolton & Britten (1955). In this, comparison of the rates of incorporation of isotope from [2-^{14}C]acetate in the absence and presence of unlabelled bicarbonate gave a more direct measure of the amounts of carbon derived from acetate or from CO_2. Brief experiments with this technique showed that, in the presence of unlabelled bicarbonate, protein of greatly reduced radioactivity was synthesized. When the results obtained were plotted as the change with time of the fraction

$$\frac{\text{total } ^{14}\text{C incorporated in the presence of unlabelled } NaHCO_3}{\text{total } ^{14}\text{C incorporated in the absence of unlabelled } NaHCO_3} \times 100$$

curves such as *A* and *B* in Fig. 1 were obtained. Since, in the period from 10 to 15 min., the rates of incorporation of ^{14}C in the presence and in the absence of unlabelled 4·3 mM-$NaHCO_3$ were

Table 1. *Radioactivity of some amino acids in protein hydrolysates from bacteria growing on [2-^{14}C]acetate in the absence and in the presence of unlabelled sodium bicarbonate*

All flasks contained *Pseudomonas* KB1 (2 mg. dry wt./ml.) in medium containing 25 mM-ammonium acetate, 25 mM-phosphate, pH 7·1, and salts. At zero time, $^{14}CH_3 \cdot CO_2Na$ was added to I, $^{14}CH_3 \cdot CO_2Na + NaHCO_3$ (final concn. 4·3 mM) was added to II and $^{14}CH_3 \cdot CO_2Na + NaHCO_3$ (final concn. 10·8 mM) was added to III. Samples (1 ml.) were taken at known times, the protein was precipitated with trichloroacetic acid, washed, hydrolysed and analysed as described under Methods.

Flask	Time (min.)	Radioactivity (counts/min.) in *iso*Leucine + leucine	Phenyl-alanine	Valine	Proline	Alanine	Threonine	Glutamate	Aspartate
I	1·5	169	46	41	25	51	38	199	195
II	1·5	67	27	28	19	24	14	78	82
III	1·5	62	24	22	18	22	11	64	68
I	5	957	296	100	171	246	213	572	650
II	5	820	178	69	133	185	190	550	539
III	5	554	168	60	84	172	138	429	456
I	10	2446	991	290	418	728	421	1533	1370
II	10	2261	833	236	391	770	412	1509	1253
III	10	2155	705	205	335	640	364	1351	1130
I	15	4767	1616	495	835	1487	1001	2986	2653
II	15	4768	1612	430	838	1457	714	3020	2756
III	15	4375	1505	358	734	1324	701	2697	2511

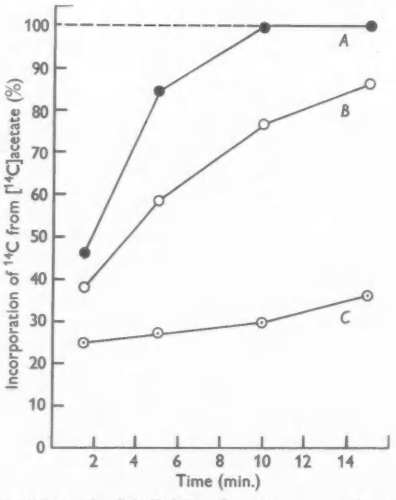

Fig. 1. Effect of unlabelled bicarbonate or pyruvate on the incorporation of ^{14}C into protein from [2-^{14}C]acetate. For conditions, see text. [2-^{14}C]Acetate was added at zero time, together with 4·3 mM-$NaHCO_3$ (curve *A*), or 10·8 mM-$NaHCO_3$ (curve *B*), or 15 mM-sodium pyruvate (curve *C*). The curves represent the variation with time of the fraction

$$\frac{\text{isotope incorporated from [2-}^{14}\text{C]acetate + unlabelled addition}}{\text{isotope incorporated from [2-}^{14}\text{C]acetate alone}} \times 100.$$

identical (Fig. 1, curve *A*), the initial lowered incorporation of ^{14}C was not due to an inhibition of protein synthesis, but is interpreted as meaning that most of the protein initially synthesized from acetate had passed through a stage readily exchangeable with CO_2. In the presence of 10·8 mM-$NaHCO_3$ (Fig. 1, curve *B*), a similar initial lowering of the radioactivity of synthesized protein was observed. After 15 min., the radioactivity of the protein in this flask had reached 85% of that synthesized in the absence of added $NaHCO_3$, again showing that the lowered initial radioactivity was not due to an inhibition of the growth of the bacteria, but indicating that protein had been derived from unlabelled precursors. A similar but more prolonged effect was noted in the presence of 15 mM-pyruvate (Fig. 1, curve *C*), suggesting that pyruvate could replace 75% of the carbon derived from acetate.

Analysis of the radioactivity of individual amino acids (Table 1) confirmed that, in the presence of unlabelled $NaHCO_3$, the protein contained less ^{14}C than in its absence. The general reduction in the radioactivity of all amino acids further suggested that the unlabelled compound synthesized from the added bicarbonate acted as precursor to all amino acids synthesized, and therefore must lie on one of the earliest stages on the pathway of acetate to protein.

Long-term incubations

The findings reported in the last section can be interpreted in two ways. They indicate either the occurrence of CO_2-fixing reactions of hitherto unsuspected magnitude, or a disturbance of the steady-state concentration, due to the addition of bicarbonate, of a compound in ready equilibrium with CO_2 lying on one of the earliest stages of the

Table 2. *Effect of carbon dioxide on the incorporation of radioactive carbon from ammonium [2-^{14}C]acetate by* Pseudomonas *KB*1 *growing on ammonium acetate at* 22°

Flask *A*, containing bacteria in 15 ml. of 50 mM-ammonium acetate growth medium, was gassed with 100% O_2 initially and stoppered tightly. Flask *B*, containing bacteria in 15 ml. of 50 mM-ammonium acetate growth medium + 100 mM-$NaHCO_3$, was gassed continuously with $CO_2 + O_2$ (5:95). $^{14}CH_3 \cdot CO_2Na$ was added at zero time. Samples (2 ml.) were removed at half-hourly intervals; the protein was hydrolysed and analysed as described under Methods.

Time (hr.)	Protein concn. (μg. of amino acid N/sample)		Total protein synthesized (μg. of amino acid N/sample)		^{14}C incorporated (counts/min./sample)		Specific radioactivity of synthesized protein (counts/min./μg. of synthesized amino acid N)	
	A	*B*	*A*	*B*	*A*	*B*	*A*	*B*
0	14	14	—	—	—	—	—	—
0·5	16	16	2	2	26 600	22 000	13 300	11 000
1·0	19	18	5	4	60 800	42 600	12 200	10 600
1·5	21	21	7	7	85 600	72 600	12 100	10 400
2·0	26	26	12	12	144 000	121 000	12 000	10 100
2·5	28	28	14	14	169 000	141 000	12 100	10 100
						Average	12 300	10 400

Table 3. *Effect of carbon dioxide on the incorporation of radioactive carbon from ammonium [2-^{14}C]acetate by* Pseudomonas *KB*1 *growing on ammonium acetate at* 30°

The conditions used were similar to those reported in Table 2.

Time (hr.)	Protein concn. (μg. of amino acid N/sample)		Total protein synthesized (μg. of amino acid N/sample)		^{14}C incorporated (counts/min./sample)		Specific radioactivity of synthesized protein (counts/min./μg. of synthesized amino acid N)	
	A	*B*	*A*	*B*	*A*	*B*	*A*	*B*
0	14	13	—	—	—	—	—	—
0·5	21	20	7	7	88 900	74 200	12 700	10 600
1·0	31	30	17	17	213 000	179 000	12 500	10 500
1·5	45	44	31	31	371 000	319 000	11 900	10 300
2·0	65	64	51	51	611 000	527 000	11 900	10 300
2·5	69	70	55	57	662 000	591 000	12 000	10 400
						Average	12 200	10 400

path from acetate to protein. Long-term experiments, in which CO_2 was continuously bubbled through the growing bacterial suspension both before and after addition of isotope, and in which there was therefore no disturbance of the steady state, support the latter explanation. At 22° (Table 2) the amino acid N concentration of samples of the protein hydrolysates rose from 14 to 28 μg./ml. in the 2·5 hr. of the incubation, both in the presence and in the absence of unlabelled CO_2. This synthesis of protein, equivalent to 14 μg. of amino acid N/sample, was accompanied by incorporations of ^{14}C from acetate, giving 169 000 counts/min./sample in the absence, and 141 000 counts/min./sample in the presence, of unlabelled CO_2. The final specific radioactivity of the protein synthesized in the two flasks was therefore 12 100 counts/min./μg. of synthesized amino acid N in the absence, and 10 100 counts/min./μg. of synthesized amino acid N in the presence, of unlabelled CO_2. The average values of the specific radioactivities, calculated from the protein synthesized and ^{14}C incorporated in all samples taken, were 12 300 and 10 400 counts/min./μg. of synthesized amino acid N respectively. When this experiment was repeated at 30° (Table 3) higher rates of both synthesis of protein and incorporation of ^{14}C were observed. The average specific radioactivities of the synthesized protein were, however, strikingly similar to those found at the lower temperature. Again, the presence of unlabelled CO_2 caused a reduction from 12 200 to 10 400 counts/min./μg. of synthesized amino acid N. These results therefore showed that, both at 22° and 30°, the presence of unlabelled CO_2 caused a reduction of 15–17% in the radioactivity of proteins synthesized from [^{14}C]acetate.

Degradation of amino acids

Degradations were carried out on amino acids synthesized in the long-term experiments. Alanine, synthesized in the absence or presence of unlabelled CO_2 by *Pseudomonas* KB1 growing on $^{14}CH_3 \cdot {}^{14}CO_2NH_4$, was uniformly labelled (Table 4). The presence of unlabelled CO_2 caused a reduction in the percentage radioactivity of the carboxyl groups of aspartic acid, as compared with the

Table 4. *Distribution of radioactive carbon in alanine and aspartic acid derived from* Pseudomonas *KB*1 *grown on ammonium* [1:2-$^{14}C_2$]*acetate in the absence and presence of carbon dioxide*

Flask *A*, containing bacteria in 15 ml. of 50 mM-ammonium acetate growth medium, was gassed with 100% O_2 initially and stoppered. Flask *B*, containing bacteria in 15 ml. of 50 mM-ammonium acetate growth medium + 100 mM-$NaHCO_3$, was gassed continually with $CO_2 + O_2$ (5:95). $^{14}CH_3 \cdot {}^{14}CO_2Na$ was added at zero time. Samples were removed after 2 hr. and the protein was hydrolysed and analysed as described under Methods. The appropriate carrier acid was added to the samples before degradation.

Amino acid	Carbon atom	Specific radioactivity of $Ba^{14}CO_3$ derived from source indicated* (counts/min./mg. of $BaCO_3$)	Percentage of total radioactivity of molecule contributed by each C atom
Alanine from flask *A*	Total†	10·7±0·2	—
	CO_2H	10·3±0·2	32·1
	$CHNH_2$	11·2±0·1	34·8
	CH_3	10·9±0·2	33·9
			Total 100·8
Alanine from flask *B*	Total†	9·2±0·2	—
	CO_2H	9·1±0·1	33·2
	$CHNH_2$	9·4±0·2	34·2
	CH_3	9·4±0·1	34·2
			Total 101·6
Aspartic acid from flask *A*	Total†	7·6±0·2	—
	CO_2H (α-)	7·4±0·1	24·2
	$CHNH_2$	—	51·1‡
	CH_2	—	
	CO_2H (β-)	7·4±0·1	24·2
Aspartic acid from flask *B*	Total†	4·1±0·1	—
	CO_2H (α-)	3·6±0·05	22·0
	$CHNH_2$	—	56·0‡
	CH_2	—	
	CO_2H (β-)	3·6±0·05	22·0

* Limits of error are expressed as the standard deviation of the specific radioactivity of triplicate samples of $Ba^{14}CO_3$.
† Determined from $Ba^{14}CO_3$ obtained from complete combustion.
‡ Calculated by difference.

Table 5. *Distribution of radioactive carbon in alanine and aspartic acid derived from* Pseudomonas *KB*1 *grown on ammonium* [2-^{14}C]*acetate in the absence of added carbon dioxide*

Amino acid samples were taken from flask *A*, as reported in Table 2, and treated similarly to those reported in Table 4.

Amino acid	Carbon atom	Specific radioactivity of $Ba^{14}CO_3$ derived from source indicated* (counts/min./mg. of $BaCO_3$)	Percentage of total radioactivity of molecule contributed by each C atom
Alanine from flask *A*	Total†	9·95±0·15	—
	CO_2H	5·25±0·1	17·6
	$CHNH_2$	12·7±0·03	42·3
	CH_3	12·5±0·1	41·9
			Total 101·8
Aspartic acid from flask *A*	Total†	15·2±0·3	—
	CO_2H (α-)	8·0±0·1	13·1
	$CHNH_2$	—	73·8‡
	CH_2	—	
	CO_2H (β-)	8·0±0·1	13·1

* Limits of error are expressed as the standard deviation of the specific radioactivity of triplicate samples of $Ba^{14}CO_3$.
† Determined from $Ba^{14}CO_3$ obtained from complete combustion.
‡ Calculated by difference.

uniform labelling observed in the absence of unlabelled CO_2. In both cases, the specific radioactivities of the individual carboxyl groups in the same molecule were equal to one another.

The labelling patterns observed in alanine and aspartic acid synthesized in the absence of added unlabelled CO_2 by *Pseudomonas* KB1 growing on $^{14}CH_3 \cdot CO_2NH_4$ are shown in Table 5. Both the α- and β-carbon atoms of alanine and the two carboxyl groups of aspartic acid are equally labelled.

DISCUSSION

The presence of carbon dioxide has long been recognized as an important factor for bacterial growth (see Krebs, 1943; Wiame & Bourgeois, 1955). That carbon dioxide can be incorporated into cellular material in non-photosynthetic organisms emerged from the work of Wood & Werkman (1935, 1936, 1938) with propionic acid bacteria. Such fixation processes might well play an important part in the synthesis of cellular constituents by micro-organisms growing on C_2 units, such as acetate. It is feasible, for instance, that C_3 units could be synthesized from carbon dioxide by mechanisms such as operate in photosynthetic (Calvin, 1955) or autotrophic organisms (Santer & Vishniac, 1955; Trudinger, 1955). This possibility is rendered unlikely, however, by the finding that phosphorylated compounds are not obligatory intermediates in the synthesis of C_4 compounds from acetate (Kornberg, 1958). Alternatively, C_3 units could be formed from condensation of acetate with carbon dioxide, or derivatives thereof. Evidence has been found for the existence of such condensations in *Clostridium kluyveri* (Tomlinson, 1954) and *Escherichia coli* (Abelson *et al.* 1954). The exchange of the carboxyl group of pyruvate with carbon dioxide has been observed in *Cl. butylicum* (Wilson, Krampitz & Werkman, 1948) and *Cl. acidurici* (Sagers & Beck, 1955). Incorporation of a C_1 unit into pyruvate occurs in extracts of *E. coli* (Utter, Lipmann & Werkman, 1945) and *Micrococcus lactilyticus* (Novelli, 1955). It could thus be envisaged that C_4 compounds are synthesized by the successive carboxylation of acetate to give pyruvate and, subsequently, malate. The latter reaction has been demonstrated in extracts of *Pseudomonas* KB1 (Kornberg & Madsen, 1958). Such consecutive carboxylation reactions would involve the incorporation from carbon dioxide of at least 50% of the cellular carbon.

The present results show that the radioactivity of bacterial protein, obtained from cells grown on [2-^{14}C]acetate in a constant stream of unlabelled carbon dioxide, is 83–85% of that obtained from cells grown on the same isotopic carbon source in contact with their own respired carbon dioxide. This shows that a major amount of protein carbon must be made from acetate units by processes not involving carbon dioxide fixation.

This conclusion is borne out by the labelling patterns of the alanine and aspartic acid synthesized from doubly-labelled acetate in the presence and absence of unlabelled carbon dioxide (Table 4). It is assumed that alanine, qualitatively at least, reflects the labelling pattern of pyruvic acid through transamination reactions. The continuous bubbling of the bacterial suspension with unlabelled carbon dioxide would result in a change of labelling pattern in any C_3 unit resulting from condensation of acetate with carbon dioxide, or derivatives thereof. No departure from the uniform labelling of alanine, as observed in flask *A*, occurs in the presence of unlabelled carbon dioxide in flask *B*. This finding rules out the occurrence of condensations of acetate with carbon dioxide as being of quantitative importance in the synthesis of cell constituents from acetate, except in the event of a C_3 compound being formed which is not in ready equilibrium with pyruvic acid and hence with alanine. No evidence for the formation of such compounds has been found (Kornberg, 1958).

The change of labelling pattern of the aspartic acid, synthesized in the presence of unlabelled carbon dioxide (Table 4), shows that a carbon dioxide-fixation reaction is involved in its formation. Since the fall of radioactivity is shared equally by both the carboxyl groups, a symmetrical precursor must be formed. This suggests that the aspartic acid is formed from fumaric acid and ammonia by the action of aspartase, an enzyme found in this bacterial species (Cook & Woolf, 1928). A similar conclusion is reached from isotopic experiments on acetate-grown *E. coli* with $^{13}CH_3 \cdot {}^{14}CO_2Na$ (Cutinelli, Ehrensvärd, Reio, Saluste & Stjernholm, 1951).

It might be expected that the fall of radioactivity noted in the carboxyl groups of aspartic acid, synthesized in the presence of unlabelled carbon dioxide, should be detected also in the carboxyl group of alanine. That this is not observed may be due to the synthesis of malic acid by two routes: (*a*) from fumaric acid by the action of fumarase and (*b*) from condensation of acetate with glyoxylate by malate synthetase (cf. Kornberg & Madsen, 1958). It seems likely that these reactions occur at different sites in the cell, and could lead to two types of labelled malic acid being formed, only one of which would be in ready equilibrium with the aspartic acid derived from fumarate.

The existence of two such pools of differently labelled malate could then prevent the complete correspondence of label in the carboxyl groups of the alanine and aspartate derived therefrom.

Aspartic acid which is formed by routes not involving carbon dioxide fixation must obviously be uniformly labelled. Since pyruvate in the presence of unlabelled carbon dioxide is shown to remain uniformly labelled, it can be calculated that one molecule of aspartic acid in every three must have arisen from carboxylation of pyruvate in order to give the observed labelling. Thus at least 8% of the total carbon incorporated into C_4 units related to oxaloacetate must come from carbon dioxide.

The labelling data are thus in accord with the measurements of the specific radioactivity of the synthesized protein in showing that a major portion of the cellular constituents must be formed by reactions other than carbon dioxide fixation.

The labelling pattern of alanine derived from *Pseudomonas* KB1 grown on $^{14}CH_3 \cdot CO_2NH_4$ in the absence of added unlabelled carbon dioxide shows the α- and β-carbon atoms to be equally labelled (Table 5), and thus that both are derived from the methyl group of acetate. A similar observation was made by Cutinelli *et al.* (1951) in acetate-grown *E. coli*. It is known that *Pseudomonas* KB1 oxidizes acetate by the reactions of the tricarboxylic cycle (Kogut & Podoski, 1953; Kornberg, 1958) and recycling of [2-^{14}C]acetate through these reactions would lead to oxaloacetic acid labelled

$$\begin{array}{cccc} HO_2C & : & CH_2 & : & CO & : & CO_2H \\ 16{\cdot}7 & : & 33{\cdot}3 & : & 33{\cdot}3 & : & 16{\cdot}7 \end{array}$$

(the figures express the percentage of the total radioactivity of the molecule contributed by each carbon atom). Pyruvic acid derived from decarboxylation of oxaloacetate would be labelled

$$\begin{array}{cccc} CH_3 & : & CO & : & CO_2H \\ 40 & : & 40 & : & 20 \end{array}$$

The observed labelling patterns (Table 5) show a lowering of radioactivity in the carboxyl groups of both alanine and aspartic acid from those values predicted for sole operation of the tricarboxylic acid cycle. Since unlabelled carbon from carbon dioxide does not significantly enter the alanine molecule, the labelling patterns of oxaloacetic and pyruvic acids derived from $^{14}CH_3 \cdot CO_2H$ labelled '100' in the methyl group would become, respectively,

$$\begin{array}{cccc} HO_2C & : & CH_2 & : & CO & : & CO_2H \\ 50 & : & 100 & : & 100 & : & 50 \end{array}$$

and

$$\begin{array}{cccc} CH_3 & : & CO & : & CO_2H \\ 100 & : & 100 & : & 50 \end{array}$$

by sole operation of the tricarboxylic cycle. The significance of the fall in radioactivity observed in the respective carboxyl groups in Table 5 is emphasized by expressing their percentage figures by the above notation, namely,

$$\begin{array}{cccc} HO_2C & : & CH_2 & : & CH(NH_2) & : & CO_2H \\ 35{\cdot}4 & : & & 200 & & : & 35{\cdot}4 \end{array}$$

and

$$\begin{array}{cccc} CH_3 & : & CH(NH_2) & : & CO_2H \\ 99 & : & 100 & : & 41{\cdot}5 \end{array}$$

This suggests the existence of some pathway leading to an enhanced incorporation of the carboxyl group of acetate into the carboxyl groups of aspartic acid and alanine. The operation of the 'glyoxylate cycle' (Kornberg & Madsen, 1957, 1958; Kornberg & Krebs, 1957), demonstrated in bacterial extracts, would lead to this effect. In this cycle, unlabelled carbon from the carboxyl group of $^{14}CH_3 \cdot CO_2H$ enters the metabolic routes not only by condensation with oxaloacetate to form citrate, but also by condensation with glyoxylate (derived from *iso*citrate) to form malate. Recycling of [2-^{14}C]acetate through these reactions would lead to oxaloacetic acid labelled

$$\begin{array}{cccc} HO_2C & : & CH_2 & : & CO & : & CO_2H \\ 0 & : & 50 & : & 50 & : & 0 \end{array}$$

and pyruvic acid labelled

$$\begin{array}{ccccc} CH_3 & : & CO & : & CO_2H \\ 50 & : & 50 & : & 0 \end{array}$$

The amount of radioactivity in the carboxyl groups of alanine and aspartic acids, which were lower than expected on the sole operation of the tricarboxylic acid cycle, but higher than expected on the sole operation of the 'glyoxylate cycle', agrees well with the view that the 'glyoxylate cycle' operates to replace intermediates drained from the tricarboxylic acid cycle during growth. The magnitude of the effect observed in the carboxyl labelling is of such an order that it would escape detection in the isotopic analyses performed by Cutinelli *et al.* (1951) on amino acids synthesized by *E. coli*.

SUMMARY

1. The effect of non-radioactive carbon dioxide on the incorporation of ^{14}C into protein synthesized from ammonium [^{14}C]acetate by *Pseudomonas* KB1 has been studied.

2. The addition of unlabelled bicarbonate or pyruvate depressed the initial rate of incorporation of isotope into protein.

3. Cells growing in a stream of unlabelled carbon dioxide synthesized protein of specific radioactivity 15–17% lower than that synthesized in the absence of added unlabelled carbon dioxide.

4. Alanine and aspartate synthesized from [^{14}C]acetate in the presence and absence of unlabelled carbon dioxide were degraded. Unlabelled carbon dioxide did not affect the distribution of isotope within the alanine molecule but caused a uniform reduction of radioactivity in the carboxyl carbons of aspartate. These findings show that carbon dioxide-fixation reactions play no part in the synthesis of alanine from acetate but participate in the formation of aspartate.

5. The observed labelling patterns provide evidence for the operation of the glyoxylate cycle in these organisms.

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The Metabolism of C_2 Compounds in Micro-organisms

3. SYNTHESIS OF MALATE FROM ACETATE VIA THE GLYOXYLATE CYCLE*

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When micro-organisms, such as *Pseudomonas*, grow on acetate as sole source of carbon, acetate enters the tricarboxylic acid cycle at two sites, to form citrate at one and a compound metabolically close to or identical with malate at the other (Kornberg, 1958; Kornberg & Quayle, 1958). The experiments described in this paper were undertaken to elucidate the intermediate steps of these reactions. The results show that cell-free extracts of *Pseudomonas* grown on acetate, which catalyse the formation of acetyl-coenzyme A from acetate, can promote the condensation of acetyl-coenzyme A both with oxaloacetate to form citrate (Ochoa, Stern & Schneider, 1951) and with glyoxylate to form malate (Wong & Ajl, 1956). Glyoxylate, which serves as substrate for the latter condensation, can be derived from the aldol fission of *iso*citrate to succinate and glyoxylate (Smith & Gunsalus, 1954; Olson, 1954; Wong & Ajl, 1955; Saz & Hillary, 1956).

In conjunction with the known reactions leading from citrate to *iso*citrate, and from malate to oxaloacetate, these reactions represent a variant of the tricarboxylic acid cycle, leading to the net synthesis of one molecule of C_4 dicarboxylic acid from two molecules of acetate. The two reactions which involve glyoxylate have been referred to as

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the 'glyoxylate by-pass' (Kornberg & Madsen, 1957*b*), and the entire cycle has been termed the 'glyoxylate cycle' (Kornberg & Krebs, 1957). This cycle is an essential link in the synthesis of cell constituents by micro-organisms growing on acetate as sole carbon source.

This work has been presented in part to the Biochemical Society (Kornberg & Madsen, 1957*a*).

METHODS

Growth of organisms. Pseudomonas fluorescens KB1 (Kogut & Podoski, 1953) was grown in 4 l. batches of the medium (containing salts, 50 mM-phosphate buffer, pH 7·2, and 50 mM-ammonium acetate) previously described (Kornberg, 1958), at 30° with aeration at the rate of approx. 3 l. of air/min. When the cells had reached a density of 0·5–0·6 mg. dry wt./ml., they were harvested by centrifuging at 1500 *g* at 0°, washed with 0·9% KCl and stored at −12°.

Escherichia coli, mutant M 22–64 (Gilvarg & Davis, 1956), was maintained on slopes containing 0·5% of glucose, 0·2% of L-glutamate, 0·7% of K_2HPO_4, 0·8% of KH_2PO_4, 1% of $(NH_4)_2SO_4$, 0·1% of $MgSO_4$ and 1·5% of agar agar (all concentrations are %, w/v).

For experimental use, cells were grown in 400 ml. of liquid medium similar to that used for agar slopes, but with the agar omitted, in Carrel culture flasks (J. A. Jobling and Co. Ltd., Sunderland) at 30° with shaking. The harvest and subsequent treatment of cells were similar to those used for *Pseudomonas* KB1.

Preparation of extracts. Batches of frozen cells were crushed at −25° in a Hughes press (Hughes, 1951). The crushed material was homogenized with 2 vol. of 0·1 M-potassium phosphate, pH 7·5, in a glass homogenizer, and the suspension was centrifuged at 25 000 *g* for 30 min. at 0°. The clear supernatant fraction was used in all experiments except when otherwise specified.

Isotopic solutions used. Solutions of [2-^{14}C]acetate, prepared as previously described (Kornberg, 1958), contained 200 μc of ^{14}C and 36 μmoles of acetate/ml., and gave $1{\cdot}4 \times 10^7$ counts/min./ml. under the conditions of radioassay used. A solution of $KH^{14}CO_3$, prepared from $Ba^{14}CO_3$ (Radiochemical Centre, Amersham, Bucks) as previously described (Kornberg, Davies & Wood, 1952), contained 200 μc of ^{14}C and 250 μmoles of $KHCO_3$/ml., and gave 8×10^6 counts/min. under the conditions of radioassay used.

Chemicals. Sodium pyruvate, sodium oxaloacetate and barium *iso*citrate, prepared according to the method of Fittig & Miller (1889) (see Deutsch & Phillips, 1957), were gifts from Professor H. A. Krebs, F.R.S. The *iso*citrate was converted into the potassium salt by removal of the barium with K_2SO_4. Samples of sodium glyoxylate were gifts from Dr D. B. Sprinson (Columbia University Medical School, New York) and from Dr I. Zelitch (Connecticut Agricultural Experimental Station, New Haven, Conn., U.S.A.). Reduced glutathione was a gift from The Distillers Co. (Biochemicals) Ltd., Speke, Lancs. Pyridine nucleotides were purchased from the Sigma Chemical Co., St Louis, Mo., U.S.A.

Other reagents were of analytical grade.

Chemical and enzyme determinations. Glyoxylate and pyruvate were measured as 2:4-dinitrophenylhydrazones by the procedure of Friedemann & Haugen (1943). The 2:4-dinitrophenylhydrazones were chromatographically identified by the procedure of El Hawary & Thompson (1953). *iso*Citrate was determined spectrophotometrically by the production of reduced triphosphopyridine nucleotide (TPNH) from triphosphopyridine nucleotide (TPN) catalysed by *iso*citric dehydrogenase. Malate was measured manometrically with the malic decarboxylase of *Lactobacillus arabinosus* (Nossal, 1951); succinate with succinic oxidase.

Acetate-activating enzyme (acetic-thiokinase) was determined as described by Jones & Lipmann (1955), and *iso*citritase by a modification of the procedure of Saz & Hillary (1956).

Incubation and subsequent treatment of extracts. For experiments on the incorporation of ^{14}C from [2-^{14}C]-acetate, the incubation mixture used contained 100 μmoles of potassium phosphate, pH 7·5, 10 μmoles of reduced glutathione, 10 μmoles of $MgCl_2$, 0·08 μmole of coenzyme A (Sigma Chemical Co., St Louis, Mo., U.S.A.), 0·1 ml. of bacterial extract, 1·8 μmoles of [2-^{14}C]acetate (containing 10 μc of ^{14}C and giving $6{\cdot}8 \times 10^5$ counts/min.), 5 or 10 μmoles of unlabelled potassium acetate, 10 μmoles of other unlabelled substrates as indicated and water to 0·9 ml. The mixture was incubated at 30° for 2–3 min., after which time were added 10 μmoles of adenosine triphosphate (ATP), tetrasodium salt (Zellstoff-Fabrik Waldhof, Pharmazeutische Abteilung, Wiesbaden, Germany) or disodium salt (Sigma Chemical Co., St Louis, Mo., U.S.A.). Incubations were continued for various lengths of time, the reactions being stopped by the addition of 3 ml. of hot absolute ethanol. The 75% (v/v) aqueous ethanolic suspensions were centrifuged, the precipitates washed with 1 ml. of 20% (v/v) ethanol and the combined supernatant solutions, evaporated to dryness at 60° under a stream of H_2SO_4-washed N_2, were analysed by chromatography and radioautography as previously described (Kornberg, 1958).

The experiments on the anaerobic net synthesis of malate from acetate with unlabelled materials (Table 3, expt. II) and on the formation of malate from pyruvate, TPNH and $KH^{14}CO_3$ (Table 5) were carried out manometrically, Warburg double side-armed flasks, gassed with N_2, being used.

RESULTS

Reactions of acetate

Suspensions of crushed *Pseudomonas* did not consume oxygen and did not incorporate ^{14}C from labelled acetate when this material was added to them, either alone or in conjunction with members of the tricarboxylic acid cycle. Enzymic assay by the method of Jones & Lipmann (1955) showed, however, that such suspensions were still capable of activating acetate, in the presence of added coenzyme A, glutathione and ATP, to form acetylcoenzyme A, which was detected in the assay by the formation of hydroxamic acid (cf. Fig. 1). The rate of formation of hydroxamic acid was linear with time, and in the extract illustrated corresponded with the activation of approx. 1·6 μmoles of acetate/hr./extract from 6 mg. dry wt. of crushed bacteria. Higher rates (up to 8 μmoles/hr./

Table 1. *Reactions of acetyl-coenzyme A with tricarboxylic acid-cycle intermediates*

The complete system contained 100 μmoles of potassium phosphate, pH 7·5, 10 μmoles of glutathione, 0·08 μmole of coenzyme A, 0·1 ml. of *Pseudomonas* extract (supernatant fraction), 10 μmoles of acetate (containing 16 μc of ^{14}C), 10 μmoles of unlabelled intermediates as indicated, 10 μmoles of ATP and water to 1 ml. Incubation was at 30° for 30 min.

Tube	Compound added	Radioactivity (counts/min.) in Malate	Fumarate	Acetyl-coenzyme A	Citrate or *iso*citrate
a	None	119	258	1385	10
b	Glyoxylate	15 660	4030	62	6
c	*iso*Citrate	43 880	1511	91	4 283
d	Succinate	84	170	1420	0
e	α-Oxoglutarate	90	210	1396	25
f	Citrate	2 420	175	1047	616
g	Oxaloacetate	810	677	120	11 806
h	Malate	208	400	980	9 700

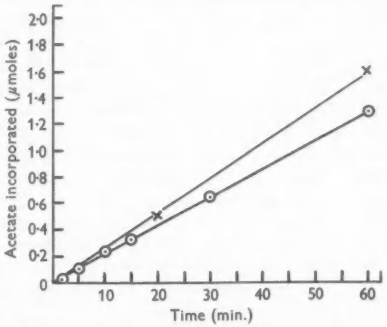


Fig. 1. Rates of acetate activation (×) and of malate formation (⊙) from acetate and glyoxylate. For measurement of acetate activation, the incubation system contained 10 μmoles of potassium acetate, 100 μmoles of potassium phosphate, pH 7·5, 10 μmoles of ATP, 0·08 μmole of coenzyme A, 10 μmoles of reduced glutathione, 10 μmoles of $MgCl_2$, 50 μmoles of KF, 200 μmoles of freshly neutralized hydroxylamine and water to 0·9 ml. *Pseudomonas* extract, supernatant fraction (0·1 ml., containing extract from 6 mg. dry wt. of crushed bacteria), was added at zero time. The quantities of hydroxamic acid formed were determined by measurement of the optical density at 540 mμ after addition of 1·5 ml. of the $FeCl_3$ reagent of Jones & Lipmann (1955). The formation of [^{14}C]malate from [2-^{14}C]acetate and glyoxylate was measured by radioassay of the labelled malate on paper-partition chromatograms (Kornberg, 1958). The incubation system used was similar to that described above but contained [2-^{14}C]acetate (0·05 ml., containing 1·8 μmoles of acetate and giving $6·8 \times 10^5$ counts/min.), 5 μmoles of unlabelled acetate and 10 μmoles of sodium glyoxylate. Fluoride and hydroxylamine were omitted.

mg. dry wt. of bacteria) were obtained with subsequent extracts. No differences were observed between suspensions of crushed bacteria and the supernatant fractions obtained therefrom; for convenience, the latter preparations were therefore used.

When [2-^{14}C]acetate was added to such supernatant fractions in the presence of added coenzyme A, glutathione and ATP, ^{14}C was incorporated chiefly into acetyl-coenzyme A, with traces in malate, fumarate, and citrate or *iso*citrate (Table 1, tube *a*). No succinate or succinyl-coenzyme A was detected, indicating that the reactions described by Seaman & Naschke (1955) in *Tetrahymena* were not present in these preparations. The presence of condensing enzyme (Ochoa *et al.* 1951) was shown by the heavy incorporation of ^{14}C into citrate on addition of oxaloacetate (Table 1, tube *g*). Considerable amounts of ^{14}C (seven times that observed in the absence of added oxaloacetate) appeared also in malate, accompanied by a trace of labelled succinate. Since it was known that *Pseudomonas* KB1 contained *iso*citritase (Saz & Hillary, 1956), this finding suggested that malate might be formed in these supernatant fractions via the actions of aconitase, *iso*citritase and the malate synthetase of Wong & Ajl (1956). That malate synthetase was present in the extracts is shown by the heavy incorporation of ^{14}C from acetate into malate in the presence of glyoxylate (Table 1, tube *b*). The combined action of *iso*citritase (1) and malate synthetase (2) is demonstrated by the similar formation of labelled malate from labelled acetate and *iso*citrate (Table 1, tube *c*):

$$\textit{iso}\text{Citrate} \rightarrow \text{succinate} + \text{glyoxylate} \qquad (1)$$

$$\begin{aligned}\text{Glyoxylate} + \text{acetyl-coenzyme A} &\rightarrow \\ &\text{malate} \ (+\text{coenzyme A})\end{aligned} \qquad (2)$$

$$\begin{aligned}\text{Sum: } \textit{iso}\text{Citrate} + \text{acetyl-coenzyme A} &\rightarrow \\ &\text{succinate} + \text{malate} \ (+\text{coenzyme A})\end{aligned}$$

The quantity of ^{14}C incorporated into malate was nearly three times higher in the presence of *iso*citrate than in the presence of glyoxylate. A similar effect has been observed in extracts of castor beans (Kornberg & Beevers, 1957). In such extracts, the addition of glyoxylate from a motor-driven Agla syringe at 2 μmoles/min., more than doubled the amount of ^{14}C incorporated into

malate than when, as in the present instance, the 10 μmoles of glyoxylate were added at the start of the incubation. These findings are consistent with the view that the presence of large amounts of glyoxylate inhibit metabolic reactions (cf. D'Abramo, Romano & Ruffo, 1957). Greater incorporation would be expected from the gradual addition of glyoxylate, as is the case when glyoxylate is produced from the enzymic fission of *iso*citrate.

Amongst the substrates tested, only glyoxylate and its precursor, *iso*citrate, led to a major incorporation of ^{14}C into malate. Succinate and α-oxoglutarate were without effect (Table 1, tubes *d* and *e*). The weak effect of citrate (Table 1, tube *f*) suggested that aconitase was present only in low concentration. This suggestion was confirmed spectrophotometrically (see later) and explains the relatively low incorporation of [^{14}C]acetate into malate on addition of oxaloacetate (Table 1, tube *g*); had aconitase been present in higher activity, more labelled malate would have been expected to be formed from the synthesized labelled citrate, and the amount of labelled succinate formed would also have been greater.

In the presence of unlabelled malate, ^{14}C from acetate was incorporated chiefly into citrate or *iso*citrate (Table 1, tube *h*). This finding could be explained by the formation of oxalocetate from malate, catalysed by the action of malic dehydrogenase, which, by condensation with labelled acetate, would lead to the formation of labelled citrate. The formation of oxaloacetate from malate in this sequence of reactions would require a concomitant reduction of another substrate. Although the nature of this substrate is not known, it is established that the overall reaction (3)

$$\text{Malate} + \text{acetyl-coenzyme A} + \text{diphosphopyridine nucleotide (DPN)} \rightarrow \text{citrate} + \text{coenzyme A} + \text{reduced DPN (DPNH)} + H^+ \quad (3)$$

can take place in biological systems and has been used in the assay of condensing enzyme (Ochoa, 1955).

In all cases, the formation of labelled malate was accompanied by the formation of labelled fumarate, showing the presence of fumarase in the extracts.

Malate synthetase

The experiments reported in Table 1 show that the extracts contain both malate synthetase, discovered in *Escherichia coli* by Wong & Ajl (1956), and the condensing enzyme of Ochoa *et al.* (1951). Both enzymes catalyse the reaction of the active methyl group of acetyl-coenzyme A with the carbonyl group of an α-oxo acid. The question arises whether these interactions are catalysed by one enzyme or by separate enzymes.

This point was tested with extracts of crushed *E. coli*, mutant M 22–64, which was known to be deficient in the condensing enzyme (Gilvarg & Davis, 1956). Such extracts rapidly catalysed the formation of labelled acetyl-coenzyme A, and its interaction with glyoxylate, to form labelled malate (Table 2), showing that malate synthetase was present. Addition of oxaloacetate instead of glyoxylate did not lead to any incorporation of ^{14}C; in particular, the citrate or *iso*citrate area of the chromatograms was devoid of radioactivity. This confirms the absence of condensing enzyme from the mutant, and shows this enzyme to be different from malate synthetase.

The formation of labelled malate from labelled acetate and glyoxylate, catalysed by the malate synthetase of *Pseudomonas* KB1, proceeded linearly with time (Fig. 1). The extract used in this experiment, derived from 6 mg. dry wt. of bacteria, catalysed the formation of 1·2 μmoles of malate/hr. This is too low to account for the observed growth of *Pseudomonas* KB1 on acetate. However, the activity of acetic-thiokinase (Jones & Lipmann, 1955) in this extract was low; comparison of the rate of formation of labelled malate with that of formation of hydroxamic acid indicates the activation of acetate to be the rate-limiting step in this reaction.

Table 2. *Incorporation of radioactive carbon from* [2-^{14}C]*acetate, catalysed by extracts of* Escherichia coli *mutant M* 22–64

The conditions of incubation were similar to those used in Table 1, but only half the quantity of [2-^{14}C]acetate was used. Incubation was for 20 min. at 30°.

Tube no.	Substrate added	Total radioactivity (counts/min.) in Acetyl-coenzyme A	Malate	Fumarate	Citrate or *iso*citrate
1	None	816	112	12	0
2	Oxaloacetate	747	81	9	0
3	Glyoxylate	76	9720	580	0
4	Glyoxylate; no coenzyme A	0	5526	540	0
5	Glyoxylate; no ATP	0	0	0	0
6	*iso*Citrate	354	700	202	0

Table 3. *Synthesis of malate from acetate*

The flasks used in Expts. I and II contained, respectively, 300 and 300 μmoles of potassium phosphate, pH 7·6, 30 and 10 μmoles of $MgCl_2$, 30 and 5 μmoles of glutathione, 0·6 and 0·16 μmole of coenzyme A, 30 and 40 μmoles of ATP, and other substrates as indicated. All flasks contained 0·5 ml. of bacterial extract and water to 3 ml. Expt. I was carried out in air, the glyoxylate being added in ten portions of 2·5 μmoles each. Expt. II was performed with Warburg manometers, gassed with N_2. All flasks were incubated at 30° for 1 hr.

		Reactants (μmoles)					Products (μmoles)			
				Potassium (+)-*iso*citrate						
Expt. no.	Flask no.	Potassium acetate	Sodium glyoxylate	Initial	Final	Decrease	Succinate	Malate	Glyoxylate*	Sum of malate + glyoxylate
I	1	30	—	—	—	—	—	—	—	—
	2	30	25	—	—	—	—	9·9	—	—
II	3	300	—	15·8	2·7	13·1	11·8	8·2	0·9	9·1
	4	—	—	31·6	16·8	14·8	13·4	1·1	9·5	10·6

* Identified by chromatography of its 2:4-dinitrophenylhydrazone, by the method of El Hawary & Thompson (1953).

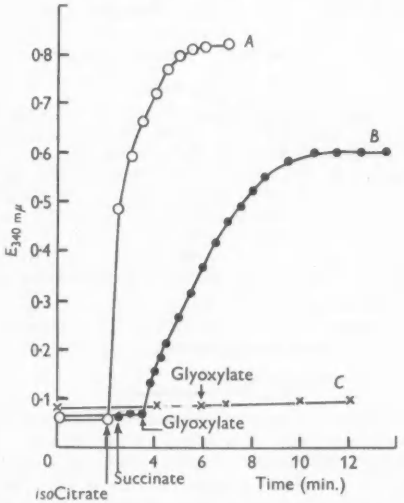


Fig. 2. *iso*Citric dehydrogenase and the reversibility of *iso*citritase. Beckman quartz cells, 1 cm. light path, contained 100 μmoles of potassium phosphate, pH 7·6, 10 μmoles of $MgCl_2$, 10 μmoles of glutathione, 0·27 μmole of TPN, 0·1 ml. of *Pseudomonas* supernatant fraction and water to 3 ml. Optical density was measured at 340 mμ. Curve *A*, 5 μmoles of *iso*citrate were added at 2 min.; curve *B*, 10 μmoles of succinate were added at 2 min. 15 sec., and 20 μmoles of glyoxylate at 3 min. 45 sec.; curve *C*, no succinate: 20 μmoles of glyoxylate were added at 6 min.

Enzymes acting on isocitrate

The bacterial extracts used contained two enzymes capable of acting on *iso*citrate. The cleavage of *iso*citrate (1), catalysed by *iso*citritase (Smith & Gunsalus, 1954; Wong & Ajl, 1955; Saz & Hillary, 1956), was demonstrated both by the formation of succinate, estimated manometrically with succinic oxidase, and by the simultaneous appearance of equal quantities of an α-oxo compound, the 2:4-dinitrophenylhydrazone of which had the spectrographic and chromatographic characteristics of glyoxylate 2:4-dinitrophenylhydrazone (Table 3).

Besides *iso*citritase, the extracts also contained an active *iso*citric dehydrogenase (Fig. 2, curve *A*) catalysing the oxidation of *iso*citrate to α-oxoglutarate, with concomitant reduction of TPN:

$$\textit{iso}\text{Citrate} + \text{TPN}^+ \rightarrow \alpha\text{-oxoglutarate} + \text{CO}_2 + \text{TPNH} + \text{H}^+ \quad (4)$$

This reaction was utilized to estimate *iso*citrate and also to demonstrate the reversibility of *iso*citritase. The addition of succinate (Fig. 2, curve *B*) or glyoxylate alone (Fig. 2, curve *C*) to the bacterial extract, to which TPN had been added, caused no change in optical density at 340 mμ and hence no reduction of the nucleotide. The addition of glyoxylate to an extract already containing succinate and TPN resulted in an immediate reduction of the nucleotide. This was due to a combination of reaction (1), acting from right to left, and reaction (4), and confirmed the reversibility of *iso*citritase (Smith & Gunsalus, 1954; Saz & Hillary, 1956).

Formation of malate from acetate and isocitrate

That the glyoxylate produced from the action of *iso*citritase on *iso*citrate (1) served as substrate for the action of malate synthetase (2) was shown by the incorporation of ^{14}C from acetate into malate (Table 1, tube *c*).

Since it was thought that the activity of acetic-thiokinase in the extracts was the rate-limiting step, the rate of formation of labelled malate was measured after pre-incubation of half the normal quantity of extract with [2-^{14}C]acetate, glutathione, ATP and coenzyme A in five times higher concentration than normally used. *iso*Citrate was added

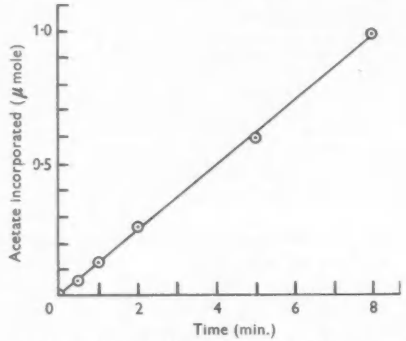


Fig. 3. Formation of [^{14}C]malate from [2-^{14}C]acetate and *iso*citrate. The incubation mixture (0·95 ml.) contained 100 μmoles of potassium phosphate, pH 7·6, 10 μmoles of glutathione, 10 μmoles of $MgCl_2$, 0·4 μmole of coenzyme A, 10 μmoles of ATP, 10 μmoles of potassium [2-^{14}C]-acetate (giving $1·4 \times 10^6$ counts/min.) and 0·05 ml. of *Pseudomonas* supernatant fraction. After pre-incubation for 15 min. at 30°, 15 μmoles of (+)-*iso*citrate were added at zero time. Samples (0·2 ml.) were removed and analysed at the times indicated.

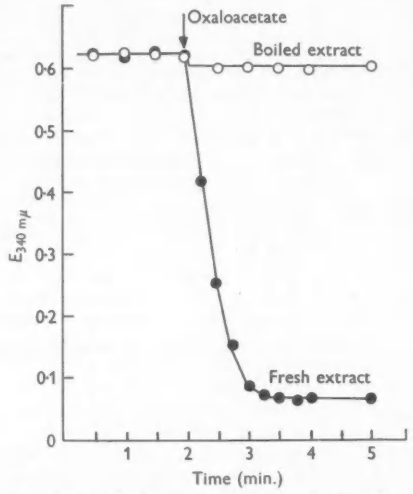


Fig. 4. Malic dehydrogenase activity of *Pseudomonas* extract. Beckman cells, 1 cm. light path, contained 60 μmoles of 2-amino-2-hydroxymethylpropane-1:3-diol (tris) buffer, pH 7·2, 3 μmoles of $MnCl_2$, 0·3 μmole of DPNH, and water to 2·9 ml. One cell (○) received 0·05 ml. of the supernatant solution obtained from boiled *Pseudomonas* extract, the other (●) received 0·05 ml. of fresh bacterial extract. Oxaloacetate (5 μmoles) was added at 2 min. Optical density was measured at 340 mμ.

after 15 min. Over the next 8 min. the incorporation of ^{14}C into malate proceeded at a linear rate (Fig. 3) corresponding to the formation of 2·5 μmoles of malate/hr./extract from 1 mg. dry wt. of bacteria. Rates of 3 μmoles/hr./extract from 1 mg. dry wt. of bacteria have been obtained. These rates are sufficiently high to account for synthesis of cell constituents by *Pseudomonas* KB 1 growing on ammonium acetate as sole carbon source.

The appearance of ^{14}C from [2-^{14}C]acetate in malate in the presence of either glyoxylate or *iso*citrate could conceivably be the result of an isotope-exchange reaction, and might not represent a net synthesis. That this is not the case is shown by the net formation of malate from acetate and *iso*citrate (Table 3). Incubation of the extract with acetate, ATP, glutathione and coenzyme A (flask 1) did not result in the appearance of malate. However, when glyoxylate was added to this system, 9·9 μmoles of malate were formed (flask 2). When *iso*citrate replaced glyoxylate in this system, 0·9 μmole of glyoxylate and 8·2 μmoles of malate were formed, accompanied by the appearance of 11·8 μmoles of succinate and the disappearance of 13·1 μmoles of *iso*citrate (flask 3). This result confirms the net synthesis of stoicheiometric amounts of malate and succinate from acetate and *iso*citrate. In the absence of added acetate (flask 4), the disappearance of 14·8 μmoles of *iso*citrate was accompanied by the appearance of 13·4 μmoles of succinate and 9·5 μmoles of glyoxylate. The formation of 1·1 μmoles of malate must be ascribed to the presence of residual acetate in the extract prepared from these acetate-grown organisms.

Reactions of malate

The bacterial extracts contained both malic dehydrogenase (Fig. 4), assayed by the oxidation of reduced DPN caused by addition of oxaloacetate, and malic enzyme (Ochoa, Mehler & Kornberg, 1948). This latter enzyme catalyses the reversible conversion of malate into pyruvate and carbon dioxide in the presence of TPN:

$$\text{Malate} + \text{TPN}^+ \rightarrow \text{pyruvate} + CO_2 + \text{TPNH} + H^+ \quad (5)$$

The stoicheiometry of this reaction was demonstrated by incubation of bacterial extract with malate and TPN. The increase in the optical density at 340 mμ observed (Table 4), which did not occur with DPN, corresponded to the reduction of 0·15 μmole of TPN in 10 min. When, at this time, the reaction was stopped by addition of trichloroacetic acid, determination of the α-oxo compounds present by the procedure of Friedemann & Haugen (1943), and chromatographic analysis of their 2:4-dinitrophenylhydrazones, showed the 0·13 μmole of pyruvate formed to be the only α-oxo compound present. The occurrence of the reverse reaction was

Table 4. *Formation of pyruvate from malate by* Pseudomonas *extract*

Two Beckman quartz cells, 1 cm. light path, each contained 100 μmoles of 2-amino-2-hydroxymethylpropane-1:3-diol (tris) buffer, pH 7·6, 3 μmoles of $MnCl_2$, 0·1 ml. of *Pseudomonas* supernatant fraction, and water to 2·9 ml. (cell *A*) or 2·7 ml. (cell *B*). TPN (1 μmole in 0·2 ml. of water) was added to cell *B*. The optical density at 340 mμ. was measured at 30-sec. intervals for 2 min., by which time no further changes were observed. Sodium L-malate (10 μmoles in 0·1 ml. of water) was added to both cells. The reaction was stopped after 5 min. by adding 0·3 ml. of 100% (w/v) trichloroacetic acid. The cell contents were centrifuged and analysed for keto compounds. The spectrum of the 2:4-dinitrophenylhydrazone was that of pyruvate. The optical density at 445 mμ of 0·1 μmole of the 2:4-dinitrophenylhydrazone of authentic pyruvate was 0·60.

Cell	Change in optical density at 340 mμ	TPN reduced (μmole)	$E_{445\,m\mu}$ for 2:4-dinitrophenyl-hydrazone	Pyruvate formed (μmole)	Pyruvate formed, corrected for blank (μmole)
A	+0·008	—	0·037	0·006	—
B	+0·324	0·15	0·82	0·135	0·13

Table 5. *Formation of malate from pyruvate and potassium* [^{14}C]*bicarbonate in the presence of reduced triphosphopyridine nucleotide*

The main compartments of three Warburg manometer flasks contained 60 μmoles of tris buffer, pH 7·6, 3 μmoles of $MnCl_2$ and 10 μmoles of unlabelled and 0·05 ml. of labelled $KHCO_3$ (giving 4×10^5 counts/min.). Flasks 1 and 3 also contained 20 μmoles of sodium pyruvate, and flasks 2 and 3 1 μmole of TPNH. The side bulbs contained 0·1 ml. of *Pseudomonas* supernatant fraction. The manometers were gassed with N_2. The contents of the side bulbs and main compartments were mixed and incubated for 30 min. at 30°. After this time, the flasks were detached and the contents pipetted into 9 ml. of ethanol at 70°. The suspensions were centrifuged and the supernatant solutions, evaporated to dryness, were analysed by two-dimensional chromatography and radioautography.

Flask no.	Contents	Radioactivity (counts/min.) in Citrate	Malate	Fumarate
1	No TPNH	90	186	38
2	No pyruvate	48	106	25
3	Complete system	42	2740	520

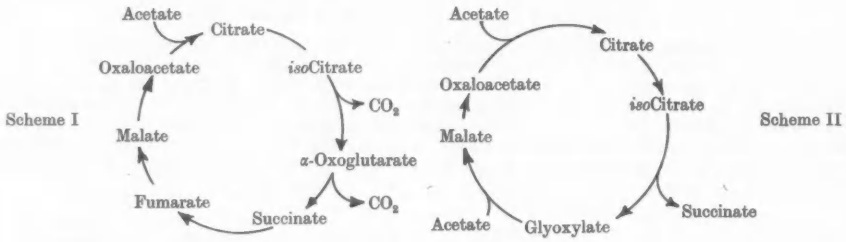
demonstrated by anaerobic incubation of bacterial extract with TPNH, pyruvate and [^{14}C]bicarbonate (Table 5). Malate and fumarate were the only labelled compounds formed, the amount of incorporation of ^{14}C into these compounds being over 20 times that observed in the absence of pyruvate or in the absence of TPNH. No ^{14}C was incorporated by the complete system containing boiled extract.

DISCUSSION

During the growth of micro-organisms, intermediates of the tricarboxylic acid cycle are utilized to provide the carbon skeletons of synthesized amino acids. When such growth occurs on simple substrates, such as acetate or ethanol as the sole sources of carbon, processes must operate to replenish from these substrates the intermediates drained from the cycle. Studies on *Pseudomonas* KB1 growing on ammonium acetate (Kornberg, 1958; Kornberg & Quayle, 1958) showed that acetate enters the tricarboxylic acid cycle at two sites, to form citrate at one and a compound identical with or metabolically close to malate at the other. These findings suggest the occurrence of reactions ancillary to the tricarboxylic acid cycle leading to the net formation of C_4 dicarboxylic acids from acetate. The number and complexity of processes occurring in whole cells make it difficult to investigate individual steps in overall reactions. Cell-free extracts were therefore used, as they neither consume oxygen nor remove malate and citrate (except under special circumstances) and allow the study of reactions leading to the net accumulation of intermediates.

The experiments reported in this paper provide evidence that acetyl-coenzyme A can react both with oxaloacetate, to form citrate, and with glyoxylate, to form malate. The aldol fission of *iso*-citrate provides the glyoxylate necessary for this latter condensation. Since the extracts also contain the enzymes capable of catalysing the conversion of citrate into *iso*citrate and of malate into oxaloacetate, it is possible to construct a modification of the tricarboxylic acid cycle (scheme I) leading to the net synthesis of C_4 dicarboxylic acids from acetate (scheme II). Kornberg & Krebs (1957), in their review of this work, proposed that this synthesizing tricarboxylic acid cycle be termed the 'glyoxylate cycle'.

Whereas the net effect of the tricarboxylic acid cycle is the combustion of one molecule of acetate to two molecules of carbon dioxide, the operation of the glyoxylate cycle, which contains only one oxidative step (that leading from malate to oxaloacetate), leads to no such loss of carbon atoms. The succinate formed is envisaged to enter the metabolic reactions of the tricarboxylic acid cycle; the glyoxylate cycle thus serves as a means



of replenishing the supply of intermediates removed in the synthesis of cell constituents. The significance of this cycle in the metabolism of bacteria, fungi and fat-containing seeds has been previously discussed (Kornberg & Madsen, 1957*b*; Kornberg & Krebs, 1957; Kornberg & Beevers, 1957).

In studies of the role of carbon dioxide fixation in growing cells (Kornberg & Quayle, 1958), no evidence was found for the existence of reactions leading to the synthesis of C_3 units from a combination of acetate with carbon dioxide, but isotope-exchange reactions were present relating C_4 compounds with C_3 units and carbon dioxide. These findings are consistent with the demonstration of 'malic enzyme' in the bacterial extracts.

SUMMARY

1. Reactions of acetate were studied in cell-free extracts of acetate-grown *Pseudomonas* KB1. Such extracts are capable of catalysing the formation of acetyl-coenzyme A from acetate.

2. Acetyl-coenzyme A thus formed reacts both with oxaloacetate, to form citrate, and with glyoxylate to form malate.

3. By the use of extracts from mutant M 22–64 of *Escherichia coli*, it has been shown that these condensations are catalysed by different enzymes.

4. The aldol fission of *iso*citrate, to succinate and glyoxylate, can provide the glyoxylate necessary for the formation of malate.

5. The cell-free extracts catalyse the net anaerobic formation of malate and succinate from acetate and *iso*citrate.

6. These reactions, in conjunction with the reactions leading from acetate to citrate, from citrate to *iso*citrate and from malate to oxaloacetate, shown to occur in the bacterial extracts, represent a synthesizing variant of the tricarboxylic acid cycle leading to the formation of one molecule of C_4 dicarboxylic acid from two molecules of acetate.

7. The rate of formation of malate from acetate and *iso*citrate is at least 3 μmoles/hr./extract from 1 mg. dry wt. of bacteria. Since malate is known to be a precursor of most cell constituents, this rate is sufficiently high to account for the observed rates of growth of *Pseudomonas* KB1 on ammonium acetate.

8. The bacterial extracts also catalyse an interconversion of malate with pyruvate and carbon dioxide that is linked with triphosphopyridine nucleotide.

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Effects of Cations on Amino Acid Decarboxylases

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Metzler, Ikawa & Snell (1954) have proposed a general mechanism for reactions catalysed by pyridoxal in which there is a requirement for a multivalent metal ion; a metal chelate of the Schiff base formed from pyridoxal phosphate and the amino acid is postulated as an intermediate. Most of their evidence in support of this theory was obtained from non-enzymic reactions of amino acids catalysed by pyridoxal which required one of the metal ions Cu^{2+}, Fe^{3+} or Al^{3+} for activity. Guirard & Snell (1954) found that the histidine decarboxylase of *Lactobacillus* 30a required pyridoxal phosphate and Fe^{3+} or Al^{3+} ions for activity, other cations being relatively ineffective or inhibitory. A requirement for Fe^{3+} ions was also found by McCullough, Piligian & Daniel (1957) for an enzyme in *Escherichia coli* O111:B4, which decarboxylated aminobenzoates in the presence of pyridoxal phosphate. Copper ions are known to inhibit many bacterial amino acid decarboxylases (Taylor & Gale, 1945).

It was therefore thought to be of interest to test whether an activation by multivalent cations is a characteristic property of other amino acid decarboxylases. The experiments described in this paper show that many bacterial decarboxylases are activated by bivalent cations.

EXPERIMENTAL

The strains of *Escherichia coli* and *Clostridium welchii*, their growth, collection and washing and the preparation of cell-free extracts were as previously described (Eggleston, 1957). Dialysis of cell-free extracts was carried out against water at 2° for 18–24 hr.

Amino acid decarboxylase activities of bacterial preparations were examined in a Warburg apparatus at 25°. In most experiments the cups contained 0·2 ml. of M-sodium acetate buffer, pH 4·5, 20 μg. of pyridoxal phosphate, 0·6–10 mg. dry wt. of enzyme; metal salt solution (where added) was in the main compartment, and 0·2 ml. of 0·25 M-L-amino acid was in the side bulb. The final volume in each cup was 2 ml. The histidine decarboxylase of *E. coli* F was found to have a higher pH optimum than the other bacterial enzymes; these experiments were therefore carried out at pH 5·5.

Extracts were made of vegetable marrow or orange fruit, by macerating the tissue in a Waring Blendor and straining the pulp through muslin, followed by dialysis against ice–water overnight. Experiments with the glutamic acid decarboxylase in citrus fruit (Axelrod, Jang & Lawrence, 1955) and in marrow were carried out in a total volume of 4 ml., at least half of which consisted of enzyme solution. The cups also contained 0·4 ml. of M-acetate buffer, pH 5·5, 20 μg. of pyridoxal phosphate and 0·2 ml. of 0·25 M-L-glutamate.

Brains from rats were ground up in a mortar with sand and 2 vol. of water and then dialysed for about 5 hr. against ice–water. Each Warburg cup contained 3 ml. of enzyme, 0·2 ml. of M-sodium phosphate buffer, pH 6·8, 20 μg. of pyridoxal phosphate and 0·2 ml. of 0·25 M-L-glutamate in a total volume of 4 ml. The gas space was filled with N_2, a freshly scraped stick of yellow phosphorus placed in the centre well and the incubation carried out at 35°.

The cations used were in the form of solutions of: KCl, Li_2SO_4, NH_4Cl, $AgNO_3$, $MgCl_2$, $MnCl_2$, $CoCl_2$, $CaCl_2$, $BaCl_2$, $NiSO_4$, $FeSO_4$, $ZnCl_2$, $CuSO_4$, $SnCl_2$, $HgCl_2$, $FeCl_3$, $AlCl_3$ ($SnCl_2$ and $FeCl_3$ were dissolved in 0·01 N-HCl; $FeSO_4$ was dissolved in water immediately before use). Unless otherwise stated, the final concentration of the salt added to the Warburg cups was mM. Each cation effect was examined at least twice with each of the enzymes tested.

RESULTS

Bacterial enzymes. The results shown in Table 1 indicate that some bivalent cations accelerate the decarboxylations of L-lysine, L-arginine, L-glutamate and L-ornithine. The largest activations (40–50%) and the most extensive (by seven cations) were found with the L-lysine decarboxylase of *E. coli* 8571. In this enzyme system, Mg^{2+}, Mn^{2+}, Co^{2+}, Ca^{2+}, Ba^{2+}, Ni^{2+} and Fe^{2+} ions were about equally effective, Al^{3+} ions about half as active and Zn^{2+}, Sn^{2+} and Fe^{3+} ions were without effect. The cations Cu^{2+}, Hg^{2+} and Ag^{+} were very strongly inhibitory, as already noted by Taylor & Gale (1945). Other strains and other decarboxylases were most frequently activated by Mg^{2+}, Mn^{2+} and Co^{2+} ions. There were considerable differences between the eight enzymes, e.g. an activation by Ca^{2+} or Fe^{2+} ions was found with only three, and by Ni^{2+} ions with only four, of these enzymes. Zinc ions had no effect where tested and in the same systems Sn^{2+} ions were inhibitory. The L-histidine decarboxylase of *E. coli* F, however, was not activated by any of the nine metal ions tested; six of these were inhibitory. The univalent cations K^{+}, Li^{+} and NH_4^{+} had little or no effect on the decarboxylations of lysine, arginine or ornithine. With seven of the eight enzymes, Fe^{3+} and Al^{3+} ions were inhibitory to varying degrees.

These activations by bivalent cations were obtained at the optimum pH of the enzymes. No activation was found at the neutral point, even when the enzymes were stimulated by the addition of inorganic phosphate (Eggleston, 1957).

Table 2 shows that the addition of Mg^{2+} ions had a greater effect in the presence of pyridoxal phosphate than in its absence. An exception to this was seen with the histidine decarboxylase, where only the coenzyme activated.

Although dialysis reduced the activity of the decarboxylases the activating effect of adding Mg^{2+} ions alone (without pyridoxal phosphate) was found to be the same as before dialysis (see Table 3).

The effect of a chelating agent, ethylenediaminetetra-acetate (EDTA) is shown in Table 4. Its inhibitory effect on lysine and glutamate decarboxylations was only small and could be reversed by the addition of extra Mg^{2+} ions.

Plant-tissue and brain glutamic decarboxylases. Dialysed extracts of vegetable marrow and orange fruit which decarboxylated L-glutamic acid were not activated by any of the cations tested. The orange enzyme was inhibited by Al^{3+} ions, and both the marrow and orange enzymes were inhibited by Fe^{2+} and Fe^{3+} ions. The bivalent cations Mg^{2+}, Mn^{2+}, Co^{2+}, Ca^{2+}, Ba^{2+} and Ni^{2+} had no effect. The dialysed brain enzyme was inhibited by Fe^{2+}, Co^{2+} and Ni^{2+} ions. Other cations had no

Table 1. *Effect of cations on bacterial amino acid decarboxylases*

The experimental conditions are described in the text. The concentration of each cation was mM. With the first three organisms dialysed cell-free extracts were used; otherwise whole cells were used. Results are average percentage changes (greater than 5%) in the rates of evolution of CO_2 in the first 30 min. of incubation at 25°. The last column shows the CO_2 output in the control, where no cation was added.

Organism	Amino acid	Cation added														Control (μl. of CO_2)
		K^+	Li^+	NH_4^+	Ag^+	Mg^{2+}	Mn^{2+}	Co^{2+}	Ca^{2+}	Ba^{2+}	Ni^{2+}	Fe^{3+}	Zn^{2+}	Cu^{2+}	Sn^{2+}	Hg^{2+}	Fe^{3+}	Al^{3+}	
E. coli N.C.I.B. 8571	L-Lysine	0	0	+12	−97	+46	+47	+40	+43	+46	+39	+39	0	−96	−6	−96	0	+21	237
E. coli F	L-Arginine	0	0	0	—	+18	+6	0	0	0	0	−10	−7	0	—	—	−73	−17	318
E. coli F	L-Histidine	—	—	—	—	—	0	−17	0	0	−7	−21	−16	—	—	—	−63	−49	122
E. coli 4	L-Arginine	—	—	—	—	+20	+11	+14	0	0	0	0	−8	—	—	—	−54	−43	195
E. coli 4	L-Lysine	—	—	—	—	+28	+12	+18	0	+5	+8	0	—	—	—	—	−67	−48	232
E. coli 4	L-Glutamate	—	—	—	—	+20	0	+13	0	+10	+11	−8	—	—	—	—	−20	−34	206
E. coli 4	L-Ornithine	0	0	0	—	+21	+19	+22	+23	+6	+10	+9	0	—	−77	—	−50	−10	91
C. welchii S.R. 12	L-Glutamate	—	—	—	—	+14	+12	+19	+11	+8	0	+9	—	—	—	—	−18	−27	280

Table 2. *Effect of magnesium ions on decarboxylases in the absence and in the presence of pyridoxal phosphate*

Dialysed cell-free extracts of *E. coli*, strain 8571 with L-lysine, strain F with L-arginine and L-histidine, were used. Pyridoxal phosphate (20 μg.) and mM-$MgCl_2$ were added; other details are as in the text. Results show μl. of CO_2 liberated/60 min. at 25°.

	Pyridoxal phosphate absent			Pyridoxal phosphate present		
	No additions	Mg^{2+} ions	Increase due to Mg^{2+} ions	Pyridoxal phosphate	Pyridoxal phosphate and Mg^{2+} ions	Increase due to Mg^{2+} ions
L-Lysine	163	215	+52	353	447	+94
L-Arginine	220	226	+ 6	316	372	+56
L-Histidine	87	86	- 1	126	121	- 5

Table 3. *Effect of dialysis on the activation of* L-*lysine decarboxylase by magnesium ions without added pyridoxal phosphate*

Cell-free extract of *E. coli* 8571; other details are as in the text, except that pyridoxal phosphate was omitted. Results are μl. of CO_2 liberated/30 min. at 25°.

	Additions		Increase due to Mg^{2+} ions
	None	Mg^{2+} ions (mM)	
Enzyme not dialysed	322	427	+105
Enzyme dialysed	237	341	+104

effect, except for Mn^{2+} and Ca^{2+}, which gave activations of about 10% in some experiments but not in others, possibly owing to variations in the removal of diffusible material. The addition of EDTA (0·0025 M) had no effect on either the plant-tissue or brain enzymes.

DISCUSSION

The experiments reported here show that activation by bivalent cations is widespread among bacterial amino acid decarboxylases which require pyridoxal phosphate. An exception is the histidine decarboxylase of *E. coli* F, which was stimulated by pyridoxal phosphate, but not by any of the cations tested. The plant-tissue and brain glutamic acid decarboxylases were not stimulated by bivalent cations, which may have been due to a sufficient amount of enzyme-bound cation, not removed by dialysis.

The effect of the cations in the bacterial systems can be accounted for by the hypothesis of Metzler *et al.* (1954). This hypothesis is given some support by the data of Table 2, which shows that the rates of decarboxylation of lysine and arginine by *E. coli* were highest when Mg^{2+} ions and the coenzyme were both present.

Other enzymes requiring pyridoxal phosphate have recently been shown to be activated by Mg^{2+} ions, e.g. heart-muscle glutamic–oxaloacetic transaminase (Happold & Turner, 1957), kynureninase of *Neurospora* (Jakoby & Bonner, 1953) and cystathionase of *E. coli* (Wijesundera & Woods, 1953). On the other hand, an activation by the univalent ions K^+, NH_4^+ and Rb^+ has been reported for the pyridoxal phosphate-requiring tryptophanase of *E. coli* (Happold & Struyvenberg, 1954). Blakley (1955) found stimulation of serine synthesis by pyridoxal phosphate in fractionated rabbit-liver preparations but no activation by cations or inhibition by EDTA, and concluded that this enzyme system may not require any metal ion.

It appears, then, that in some enzyme systems pyridoxal phosphate acts in conjunction with either multi- or uni-valent cations, whereas in others its action may be independent of cations.

SUMMARY

1. The activity of seven amino acid decarboxylases in *Escherichia coli* and *Clostridium welchii* acting upon L-lysine, L-arginine, L-ornithine and L-glutamate was increased (up to about 45%) by some or all of the bivalent cations Mg^{2+}, Mn^{2+}, Co^{2+}, Ca^{2+}, Ba^{2+}, Ni^{2+} and Fe^{2+}. The activation with Mg^{2+} ions was greater in the presence than in

Table 4. *Effect of ethylenediaminetetra-acetate on amino acid decarboxylases in dialysed bacterial extracts*

Dialysed cell-free extracts of *E. coli*, strain 8571 with L-lysine, strain F with L-glutamate, were used; other details are as in the text. Results are μl. of CO_2 liberated in 30 min. (with L-lysine), in 90 min. (with L-glutamate), at 25°.

	Additions			
	None	Mg^{2+} ions (mM)	EDTA (mM)	EDTA (mM) + Mg^{2+} ions (2 mM)
L-Lysine	125	202	99	208
L-Glutamate	307	350	285	340

the absence of pyridoxal phosphate. A histidine decarboxylase of *Escherichia coli* F was stimulated by pyridoxal phosphate but not by any of the nine metal ions tested.

2. Ethylenediaminetetra-acetate had only a small inhibitory effect, which was reversed by addition of Mg^{2+} ions.

3. With the same group of enzymes, K^+, Li^+ and NH_4^+ ions had little or no effect and, except with lysine decarboxylase of *Escherichia coli* 8571, the tervalent ions Fe^{3+} and Al^{3+} were inhibitory.

4. The L-glutamic acid decarboxylases of vegetable marrow, orange fruit or rat brain were not stimulated by cations nor inhibited by ethylenediaminetetra-acetic acid.

5. The results are discussed in relation to the hypothesis of Metzler *et al.* (1954).

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